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(54) Title: MINIATURIZED CELL ARRAY METHODS AND APPARATUS FOR CELL-BASED SCREENING

(57) Abstract

The present invention describes novel methods for making a substrate for selective cell patterning, and the substrates themselves, wherein the method comprises contacting reactive hydroxyl groups on the surface of a substrate with a hydroxyl-reactive bifunctional molecule to form a monolayer, and using stencils to deposit cell repulsive or cell adhesive moieties in controlled locations on the cell culture substrate. Methods comprising selective differentiation of stem cells to create tissue specific and organ-specific cell substrates, as well as the cell substrates themselves are also provided.

Oxidized plastic or clean glass has hydroxyl groups on the surface

React substrate with an aminosilane

Apply stencil

Vapor or solution deposit tresyl-PEG

Remove mechanical mask

Apply cells, they will adhere to previously masked areas

|||| Hydroxyl groups   Cell adhesive molecules

Aminosilane

Tresyl PEG

Miniaturized Cell Array Methods and Apparatus for Cell-Based Screening

Related Applications

This application claims priority to U.S. Provisional Patent Application Serial Nos. 60/127,339 filed April 1, 1999 and 60/138,119 filed June 7, 1999; is a continuation-in-part of U.S. Patent Application S/N 09/401,212, filed September 22, 1999, which is a continuation in part of U.S. Patent Application S/N 08/865,341 filed May 29, 1997; and is related to U.S. Patent Application Serial Nos. 09/468,673 filed 12/21/99.

Field of the Invention

The present invention relates to methods and devices for cell-based high throughput and high biological content screening.

Background of the Invention

In the expanding arena of drug discovery and combinatorial chemistry to generate candidate compounds, it would be very useful to be able to rapidly screen a large number of substances, via a high throughput screen, for their physiological impact on animals and humans. Before testing the efficacy of a "partially qualified" drug candidate on animals, the drug could first be screened for its biological activity and potential toxicity with living cells. The physiological response to the drug candidate could then be anticipated from the results of these cell screens.

Traditionally, "lead compounds" have moved quickly to extensive animal studies that are both time-consuming and costly. Furthermore, extensive drug testing in animals is becoming less culturally acceptable. Screening drug candidates according to their interaction with living cells, prior to animal studies, can reduce the number of animals required in subsequent drug screening processes by eliminating some drug candidates before going to animal trials. However, manipulation and analysis of drug-cell interactions using current methods does not allow for both high throughput and high biological content screening, due

High throughput screening of nucleic acids and polypeptides has been achieved using DNA chip technologies. In typical DNA analysis methods, DNA sequences of 10 to 14 nucleotides are attached in defined locations (or spots), up to tens of thousands in number, on a small glass plate. (U.S. Patent No. 5,556,752, hereby incorporated by reference). This

5   creates an array of spots of DNA on a given glass plate. The location of a spot in the array provides an address for later reference to each spot of DNA. The DNA sequences are then hybridized with complementary DNA sequences labeled with fluorescent molecules. Signals from each address on the array are detected when the fluorescent molecules attached to the hybridizing nucleic acid sequences fluoresce in the presence of light. These devices have

10 been used to provide high throughput screening of DNA sequences in drug discovery efforts and in the human genome sequencing project. Similarly, protein sequences of varying amino acid lengths have been attached in discrete spots as an array on a glass plate. (U.S. Patent 5,143,854, incorporated by reference herein).

The information provided by an array of either nucleic acids or amino acids bound to

15 glass plates is limited according to their underlying "languages". For example, DNA sequences have a language of only four nucleic acids and proteins have a language of about 20 amino acids. In contrast, a living cell, which comprises a complex organization of biological components, has a vast "language" with a concomitant multitude of potential interactions with a variety of substances, such as DNA, RNA, cell surface proteins,

20 intracellular proteins and the like. Because a typical target for drug action is with and within the cells of the body, cells themselves provide an extremely useful screening tool in drug discovery when combined with sensitive detection reagents. It thus would be most useful to have high throughput, high content screening devices to provide high content spatial information at the cellular and subcellular level as well as temporal information about

25 changes in physiological, biochemical and molecular activities.

Microarrays of cells

Methods have been described for making micro-arrays of a single cell type on a common substrate for the

which a glass plate is uniformly coated with a photoresist and a photo mask is placed over the photoresist coating to define the "array" or pattern desired. Upon exposure to

light, the photoresist in the unmasked areas is removed. The entire photolithographically defined surface is uniformly coated with a hydrophobic substance, such as an organosilane, that binds both to the areas of exposed glass and the areas covered with the photoresist. The photoresist is then stripped from the glass surface, exposing an array of spots of exposed glass. The glass plate then is washed with an organosilane having terminal hydrophilic groups or chemically reactable groups such as amino groups. The hydrophilic organosilane binds to the spots of exposed glass with the resulting glass plate having an array of hydrophilic or reactable spots (located in the areas of the original photoresist) across a hydrophobic surface. The array of spots of hydrophilic groups provides a substrate for non-specific and non-covalent binding of certain cells, including those of neuronal origin (Kleinfeld et al., J. Neurosci. 8:4098-4120, 1988).

In another method based on specific yet non-covalent interactions, stamping is used to produce a gold surface coated with protein adsorptive alkanethiol. (U.S. Patent No. 5,776,748; Singhvi et al., Science 264:696-698, 1994). The bare gold surface is then coated with polyethylene-glycol-terminated alkanethiols that resist protein adsorption. After exposure of the entire surface to laminin, a cell-binding protein found in the extracellular matrix, living hepatocytes attach uniformly to, and grow upon, the laminin coated islands (Singhvi et al. 1994). An elaboration involving strong, but non-covalent, metal chelation has been used to coat gold surfaces with patterns of specific proteins (Sigal et al., Anal. Chem. 68:490-497, 1996). In this case, the gold surface is patterned with alkanethiols terminated with nitriloacetic acid. Bare regions of gold are coated with tri(ethyleneglycol) to reduce protein adsorption. After adding Ni^{2+}, the specific adsorption of five histidine-tagged proteins is found to be kinetically stable.

More specific single cell-type binding can be achieved by chemically crosslinking specific molecules, such as proteins, to reactable sites on the patterned substrate. (Aplin and Hughes, Analyt. Biochem. 113:144-148, 1981). Another elaboration of substrate patterning optically creates an array of reactable spots. A glass plate is washed with an organosilane that chemisorbs to the glass to coat the glass. The organosilane coating is irradiated by deep

... ... causes the Si radicals t ... polar silanol groups. The polar silanol groups constitute spots on the array and are further modified to couple other reactable molecules to the spots, as disclosed in U.S. Patent

No. 5,324,591, incorporated by reference herein. For example, a silane containing a biologically functional group such as a free amino moiety can be reacted with the silanol groups. The free amino groups can then be used as sites of covalent attachment for biomolecules such as proteins, nucleic acids, carbohydrates, and lipids. The non-patterned covalent attachment of a lectin, known to interact with the surface of cells, to a glass substrate through reactive amino groups has been demonstrated (Aplin & Hughes, 1981). The optical method of forming a micro-array of a single cell type on a support requires fewer steps and is faster than the photoresist method, (i.e., only two steps), but it requires the use of high intensity ultraviolet light from an expensive light source.

In all of these methods, the result is a micro-array of a single cell type, since the biochemically specific molecules are bound to the micro-patterned chemical array uniformly. In the photoresist method, cells bind to the array of hydrophilic spots and/or specific molecules attached to the spots which, in turn, bind cells. Thus cells bind to all spots in the array in the same manner. In the optical method, cells bind to the array of spots of free amino groups by adhesion. There is little or no differentiation between the spots of free amino groups. Again, cells adhere to all spots in the same manner, and thus only a single type of cell interaction can be studied with these cell arrays because each spot on the array is essentially the same as another. Such cell arrays are inflexible in their utility as tools for studying a specific variety of cells in a single sample or a specific variety of cell interactions. Thus, there exists a need for arrays of multiple cell types on a common substrate, in order to increase the number of cell types and specific cell interactions that can be analyzed simultaneously, as well as methods of producing these micro-arrays of multiple cell types on a common substrate, in order to provide for high throughput and high biological content screening of cells.

Optical reading of cell physiology

Performing a high throughput screen on many thousands of compounds requires parallel handling and processing of many compounds and assay component reagents.

... microplates with ... of 384 wells (Kam et al., J. Biomol. Scr. 2:33-40, 1997). The signal measured from each well, either fluorescence emission, optical density, or radioactivity,

integrates the signal from all the material in the well giving an overall population average of all the molecules in the well. This type of assay is commonly referred to as a homogeneous assay.

5 U.S. Patent No. 5,581,487 describes an imaging plate reader that uses a CCD detector (charge-coupled optical detector) to image the whole area of a 96 well plate. The image is analyzed to calculate the total fluorescence per well for homogeneous assays.

Schroeder and Neagle describe a system that uses low angle laser scanning illumination and a mask to selectively excite fluorescence within approximately 200 microns of the bottoms of the wells in standard 96 well plates in order to reduce background when
10 imaging cell monolayers. (J. Biomol. Scr. 1:75-80, 1996). This system uses a CCD camera to image the whole area of the plate bottom. Although this system measures signals originating from a cell monolayer at the bottom of the well, the signal measured is averaged over the area of the well and is therefore still considered a homogeneous measurement, since it is an average response of a population of cells. The image is analyzed to calculate the total
15 fluorescence per well for cell-based homogeneous assays.

Proffitt et. al. (Cytometry 24:204-213, 1996) describe a semi-automated fluorescence digital imaging system for quantifying relative cell numbers *in situ*, where the cells have been pretreated with fluorescein diacetate (FDA). The system utilizes a variety of tissue culture plate formats, particularly 96-well microplates. The system consists of an epifluorescence
20 inverted microscope with a motorized stage, video camera, image intensifier, and a microcomputer with a PC-Vision digitizer. Turbo Pascal software controls the stage and scans the plate taking multiple images per well. The software calculates total fluorescence per well, provides for daily calibration, and configures for a variety of tissue culture plate formats. Thresholding of digital images and the use of reagents that fluoresce only when
25 taken up by living cells are used to reduce background fluorescence without removing excess fluorescent reagent..

A variety of methods have been developed to image fluorescent cells with a microscope and extract information about the spatial distribution and temporal changes

and temporal resolution imaging measurements of distribution, amount and biochemical environment of the fluorescent reporter molecules in the cells.

Treating cells with dyes and fluorescent reagents, imaging the cells, and engineering the cells to produce a fluorescent reporter molecule, such as modified green fluorescent protein (GFP), are useful detection methods (Wang et al., In Methods in Cell Biology, New York, Alan R. Liss, 29:1-12, 1989). The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has an excitation maximum at 395 nm, an emission maximum at 510 nm and does not require an exogenous factor. Uses of GFP for the study of gene expression and protein localization are discussed in Chalfie et al., Science 263:802-805, 1994. Some properties of wild-type GFP are disclosed by Morise et al. (Biochemistry 13:2656-2662, 1974), and Ward *et al.* (Photochem. Photobiol. 31:611-615, 1980). An article by Rizzuto *et al.* (Nature 358:325-327, 1992) discusses the use of wild-type GFP as a tool for visualizing subcellular organelles in cells. Kaether and Gerdes (FEBS Letters 369:267-271, 1995) report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng (FEBS Letters 369:331-334, 1995), while GFP expression in Drosophila embryos is described by Davis et al. (Dev. Biology 170:726-729, 1995). U. S. Patent No. 5,491,084, incorporated by reference herein, discloses expression of GFP from *Aequorea victoria* in cells as a reporter molecule fused to another protein of interest. Mutants of GFP have been prepared and used in several biological systems. (Hasselhoff et al., Proc. Natl. Acad. Sci. 94:2122-2127, 1997; Brejc et al., Proc. Natl. Acad. Sci. 94:2306-2311, 1997; Cheng et al., Nature Biotech. 14:606-609, 1996; Heim and Tsien, Curr. Biol. 6:178-192, 1996; Ehrig et al., FEBS Letters 367:163-166, 1995).

The ARRAYSCAN™ System, as developed by Cellomics, Inc. (U.S. Patent No. 5,989,835) and U.S. Application Serial No. 09/031,271 filed February 27, 1998; both incorporated by reference herein in their entirety) is an optical system for determining the distribution, environment, or activity of luminescently labeled reporter molecules on or in cells for the purpose of screening large numbers of compounds for specific biological

and temporal resolution imaging measurements of distribution, amount and biochemical environment of the fluorescent reporter molecules in the cells.

converting the optical information into digital data, and utilizing the digital data to determine the distribution, environment or activity of the luminescently labeled reporter molecules in

the cells. The ARRAYSCAN™ System includes apparatus and computerized method for processing, displaying and storing the data, thus augmenting drug discovery by providing high content cell-based screening, as well as combined high throughput and high content cell-based screening, in a large microplate format.

5

Microfluidics

Efficient delivery of solutions to an array of cells attached to a solid substrate is facilitated by a microfluidic system. Methods and apparatus have been described for the precise handling of small liquid samples for ink delivery (U.S. Patent No. 5,233,369; U.S.

10   Patent No. 5,486,855; U.S. Patent No. 5,502,467), biosample aspiration (U.S. Patent No. 4,982,739), reagent storage and delivery (U.S. Patent No. 5,031,797), and partitioned microelectronic and fluidic device array for clinical diagnostics and chemical synthesis (U.S. Patent No. 5,585,069). In addition, methods and apparatus have been described for the formation of microchannels in solid substrates that can be used to direct small liquid samples

15   along the surface (U.S. Patent No. 5,571,410; U.S. Patent No. 5,500,071; U.S. Patent No. 4,344,816,).

For purposes of integrated high throughput and high content cell based screening, particularly for live-cell imaging, an optimal microfluidic device would comprise a fluidic architecture that permits the closest possible well spacing (i.e.: highest possible well density),

20   wherein the fluidic architecture is integrated with the cell array substrate to permit efficient fluid delivery to the cells, and eliminating the need for pipetting fluids in and out of wells. Such optimal microfluidic devices would be advantageous for cell arrays with sub-millimeter inter-well distances because it is unwieldy, if not impossible, to pipette fluids with such a high degree of spatial resolution and accuracy. Furthermore, such integrated devices could

25   be directly used for cell based screening, without the need to remove the cell substrate from the fluidic architecture for imaging the cells.

An optimal microfluidic device for cell based screening might further comprise a closed chamber to permit environmental control of the cells. and preferably would not

polar solvents (Marc Madou, Fundamentals of Microfabrication, CRC Press, Boca Raton,

1997, p. 433). Electro-osmosis is typically accompanied by some degree of electrophoretic separation of charged medium components, such as proteins.

U.S. Patent No. 5,603,351 ('the 351 patent') describes a microfluidic device that uses a multilevel design consisting of two upper levels with channels and a bottom level with reaction wells. However, this device is not designed for use in cell based screening. The '351 patent does not disclose a substrate containing cells or cell binding sites. The disclosed microfluidic network is designed to allow two or more reagents to be combined in a reaction well, as opposed to an optimal cell screening microfluidic system that allows living cells cultured on the well bottoms to be exposed in serial fashion to two or more different fluids. The '351 patent discloses a device with the wells etched into the substrate at a maximal well density of 50 wells/inch^2. Furthermore, the substrate must be detached from the fluidic array for incubation and/or analysis. Finally, the '351 patent discloses a system of electrically-controlled electrohydrodynamic valves within the matrix of the wells that are less effective with aqueous media used in cell culture, and also may limit the degree of close-spacing between wells in the array of wells.

U.S. Patent No. 5,655,560 discloses a clog-free valving system, comprising a fluid distribution system with multiple inputs and multiple outputs incorporating a crossed array of microchannels connected vertically at crossing points by teflon valves. However, this patent does not disclose a substrate containing a cell array, nor an integrated fluidic device in combination with the substrate, nor a well density that is optimal for cell-based screening.

U.S. Patent 5,900,130 (the '130' patent) describes the active, electronic control of fluid movement in an interconnected capillary structure. This patent does not teach a fluidic architecture that maximizes the area of the cell substrate that can be occupied by cell binding sites. Nor does this patent disclose a substrate containing a cell array, nor an integrated fluidic device in combination with the substrate. Furthermore, the patent only teaches the control of fluid flow by application of an electric field to the device.

U.S. Patent 5,910,287 describes multi-well plastic plates for fluorescence measurements of biological and biochemical samples, including cells, limited to plates with

chamber to permit environmental control of the cells on the substrate.

Thus, none of these prior microfluidic devices provide a fluidic architecture that permits the closest possible well spacing (i.e.: highest possible well density), wherein the fluidic architecture is integrated with the cell array substrate to permit efficient fluid delivery to the cells, and thus eliminating the need for pipetting fluids in and out of wells.

5 Furthermore, prior microfluidic devices that comprise an array of wells use electrically-controlled electrohydrodynamic valves within the matrix of the wells that would be less effective if used with aqueous media for cell culture, and which limit the well density.

While the above advances in cell array, optical cell physiology reading, and microfluidic technologies provide supporting technologies that can be applied to improved

10 high throughput and high content cell-based screening, there remains a need in the art for integrated devices and methods that further decrease the amount of time necessary for such screening, as well as for devices and methods that further improve the ability to conduct high throughput and high content cell-based screening and the ability to flexibly and rapidly switch from one to the other. In particular, devices and methods that maximize the well

15 density, thereby increasing the number of wells that can be imaged in at one time, and thus greatly increasing the throughput of a screen while maintaining adequate resolution of the image, would be very advantageous.

The drug discovery industry already uses 96- and 384-well microplates and is in transition towards the use of 1536-well plates. However, further increases in well density

20 using prior technology are unlikely because of the great difficulty of pipetting liquids in and out of very small diameter wells.

Summary of the Invention

The present invention fulfills the need in the art for devices and methods that

25 decrease the amount of time necessary to conduct cell-based screening, and specifically combines the ability to conduct high throughput and high content cell-based screening and to flexibly and rapidly switch from one to the other The invention provides devices and methods for maximizing the number of wells that can be imaged at one time while still

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fluid exchange, and with wells that are closely spaced to more rapidly detect spatially-resolved features of individual cells.

In one aspect, the present invention provides a cassette for cell screening, comprising a substrate having a surface containing a plurality of cell binding locations; a fluid delivery system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the substrate, wherein the multi-level chamber comprises a crossed array of microfluidic input channels and output channels and a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels; and a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location, and wherein the wells are present at a density of at least about 20 wells per square centimeter.

In another aspect, the present invention provides a cassette for cell screening comprising

a.   a substrate having a surface, wherein the surface contains a plurality of cell binding locations;

b.   a fluid delivery system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the substrate, wherein the multi-level chamber comprises

i.   a crossed array of microfluidic input channels and output channels, wherein each well is in fluid connection with one or more input channels and one or more output channels;

ii.   a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels;

iii.   one or more input manifolds in fluid connection with the microfluidic input channels;

iv.   one or more output manifolds in fluid connection with the microfluidic output channels;

v.   at least one source receptacle in fluid connection with the one or more output manifolds; and

    c.   a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location.

In preferred embodiments, both of the above cassettes further comprise a pump to control fluid flow within the microfluidic device; a temperature controller of the substrate and/or a controller to regulate oxygen and carbon dioxide partial pressures.

In another aspect, the present invention provides an improved method for diffusion control in a cassette, wherein the improvement comprises constantly applying a passive restoring force to valves located within microfluidic channels of the cassette.

In a still further aspect, the present invention provides a method for cell screening, comprising

    a)   providing an array of locations which contain multiple cells;

    b)   providing an optical system to obtain images of the array of locations;

    c)   serially imaging sub-arrays of the array of locations; and

    d)   acquiring data from each of the sub-arrays in parallel.

In a preferred embodiment, the array of locations is provided as a cassette, such as those disclosed above.

In a further aspect, the present invention provides novel methods for making a substrate for selective cell patterning, and the substrates themselves, wherein the method comprises contacting reactive hydroxyl groups on the surface of a substrate with a hydroxyl-reactive bifunctional molecule to form a monolayer, and using stencils to deposit cell repulsive or cell adhesive moieties to the substrate. In preferred embodiments the hydroxyl-reactive bifunctional molecule is an aminosilane and the cell repulsive moiety comprises tresyl-activated polyethylene glycol.

Among other uses, the devices and methods of the present invention are ideal for high content and/or high throughput cell-based screening. The device of the invention is also ideally suited as a cell support system for a hand-held diagnostic device (i.e.: a miniaturized imaging cell-based assay system). The smaller format and sealed containment of the present device enables its use in a rugged, portable system. There is a great economic advantage from the ...

... stances between adjacent wells are not minimized. The present invention provides both of these advantages.

Brief Description of the Figures

Figure 1A is a top view of a small substrate micro-patterned chemical array.

Figure 1B is a top view of a large substrate micro-patterned chemical array.

5   **Figure 2** diagrams a method of producing a micro-patterned chemical array on a substrate.

Figure 3A is a photograph showing fibroblastic cell growth on a surface patterned chip, attached to a micro-patterned chemical array and labeled with two fluorescent probes.

Figure 3B is a photograph showing fibroblastic cell growth in spotted patterns, attached to a micro-patterned chemical array and labeled with two fluorescent probes.

10   **Figure 4** is a diagram of the cassette which is the combination of the micro-array of multiple cell types top and chamber bottom.

Figure 5 is a diagram of a chamber that has nanofabricated input channels to address "wells" in the non-uniform micro-patterned array of cells.

Figure 6 is a diagram of a chamber with no channels.

15   **Figure 7A** is an overhead diagram of a chamber with microfluidic channels etched onto the substrate.

Figure 7B is a side view diagram of a chamber with microfluidic channels etched onto the substrate.

Figure 8A is an overhead diagram of a chamber where the microfluidic channels and wells

20   are formed from a raised matrix of a material stamped onto the fluid delivery chamber.

Figure 8B is a side view diagram of a chamber where the microfluidic channels and wells are formed from a raised matrix of a material stamped onto the fluid delivery chamber.

Figure 9 is a diagram of a chamber where each well is addressed by a channel originating from one side of the chamber.

25   **Figure 10** is a diagram of a chamber where the wells are addressed by channels originating from two sides of the chamber.

Figure 11 is a diagram of a chamber where the microfluidic switches are controlled by light, heat or other mechanical means.

Figure 12

Figure 14A is a flow chart providing an overview of the cell screening method.

Figure 14B is a Macro (High Throughput Mode) Processing flow chart.

Figure 14C is a Micro (High Content Mode) Processing flow chart.

Figure 15 is a diagram of the integrated cell screening system.

Figure 16 is a photograph of the user interface of the luminescence reader instrument.

Figure 17A is a photograph showing lymphoid cells nonspecifically attached to an unmodified substrate.

Figure 17B is a photograph showing lymphoid cells nonspecifically attached to an IgM-coated substrate.

Figure 17C is a photograph showing lymphoid cells specifically bound to a whole anti-serum-coated substrate.

Figure 18A is a photographic image from High Throughput Mode of luminescence reader instrument identifying "hits".

Figure 18B is a series of photographic images showing the high content mode identifying high content biological information.

Figure 19 is a photographic image showing the display of cell data gathered from the high content mode.

Figure 20 depicts the optimization of cell imaging by simultaneous imaging of a sub-array of cell binding locations of the whole array. There is a one to one correspondence between the array of cell binding locations on the substrate, the array of fluidic locations on the chamber, and the array of wells formed by the joining of the two.

Figure 21 exemplifies an 8 x 8 array (64 wells, 128 channels) of the microfluidic device wherein each fluidic location is provided with a separate input and output channel, yielding $2n^2$ channels for an n x n array.

Figure 22 depicts the microfluidic device of Figure 21 wherein $m + n^2$ valves or pumps are utilized to control the flow of m liquid or vapor mixtures to an 8 x 8 array.

Figure 23 depicts an embodiment of the microfluidic device wherein the fluid delivery system consists of a crossed, nonintersecting array of input and output channels, and horizontal connecting channels utilizing multiple levels, and wherein the two layers lie in the same plane, and

Figure 25 depicts an embodiment of the microfluidic device wherein the fluid delivery system consists of a crossed, nonintersecting array of input and output channels, and vertical

connecting channels utilizing multiple levels, and wherein the two layers lie in a plane above that of the well layer.

Figure 26 depicts an end view and side view of the embodiment of Figure 25.

Figure 27 shows an embodiment of the microfluidic device wherein compounds are multiplexed to a single fluidic location by using m positive pressure source reservoirs or pumps, a valve-less manifold, and valve-free waste reservoir at atmospheric pressure.

Figure 28 shows an embodiment of the microfluidic device wherein compounds are multiplexed to a single fluidic location by using m positive pressure source reservoirs or pumps, a valved manifold, or a negative pressure waste reservoir or pump with m valves connected to m source reservoirs at atmospheric pressure and valve-free waste reservoir at atmospheric pressure.

Figure 29 shows an embodiment of the microfluidic device wherein the array of m positive pressure source reservoirs or pumps is multiplexed to an $n \times n$ crossed channel array of fluidic locations by means of $1 \times n$ input and $n \times 1$ output valve manifolds.

Figure 30 shows an embodiment of the microfluidic device with m reservoirs at atmospheric pressure and a negative pressure waste reservoir by means of $1 \times n$ input and $n \times 1$ output valve manifolds, where the negative pressure reservoir may be either mechanically or thermodynamically pumped.

Figure 31 shows an embodiment a dual-pumped system, in which there is only one waste negative pressure reservoir that must be actuated in coordinated fashion with the actuation of any particular positive pressure source reservoir. This negative pressure reservoir may function by either mechanical or thermodynamic (e.g., a capillary action pump) means. For the capillary action pump, the "actuation" of the pump is acheived by means of valves.

Figure 32 shows how the pumping and valving scheme of Figure 29, employing positive pressure source reservoirs, can be extended to work with a crossed array of n row channels and $2n$ column channels (n ordinary column channels plus n rinse column channels). This requires a $2n \times 1$ output valve manifold. Similarly, the scheme of Figure 30, employing a negative pressure waste reservoir, can be extended to work with an array incorporating rinse channels

Figure 34 depicts an embodiment of the microfluidic device wherein the fluid delivery system consists of a crossed, nonintersecting array of input and output channels with vertical connecting channels, utilizing multiple levels, wherein the two layers lie in a plane above that of the well layer and <u>every</u> column channel k has an associated parallel rinse channel k^*.

Figure 35 shows, in a case of fluid flow through well (j,k), an example of a method to rinse a segment of liquid or vapor from row channel j and into rinse channel k^*, where k^* is immediately adjacent to column channel k.

Figure 36 depicts a method of diffusion control using "normally-closed" check valves, comprising a valve element and a valve seat, between every pair of adjacent vias that connect the channels to the fluidic locations.

Figure 37 depicts an embodiment wherein an externally-applied magnetic field gradient induces a restoring force on valve elements and valve seat that incorporate a magnetic material.

Figure 38 shows an embodiment of a shaped valve seat wherein diffusion past the ball is stopped when the externally-applied restoring force presses the ball to the left into a round opening in the valve seat.

Figure 39 shows an embodiment that allows a single magnetic field gradient applied to the whole microfluidic device to induce force on both a set of ball valves of the input channels and a set of ball valves of the output channels

Figure 40 shows one embodiment of the pumping and valving schemes of this invention as it would appear with all the pumps and valves on-board the cassette.

Figure 41 shows an embodiment, wherein the pumps are on-board and utilize electrically-driven flow, where the electric fields are limited to regions external to the matrix of the array of wells, and minimal or no electric field gradients are applied across any of the wells in which live cells are present.

Figure 42 (a) The standard structure of tresyl-PEG, wherein "n" can equal any number. **(b)** The structure of trimethoxysilylpropyldiethylenetriamine.

Figure 43 (a) An example of an amine-PEG surface product. **(b)** An example of a surface

Figure 45 is an experimental flowchart for selective differentiation of stem cells on a substrate to produce both tissue-specific and organ-specific cell substrates.

GUIDE TO THE FIGURES

5

001	fluidic location
002	array of fluidic locations, optionally with depressions
003	sub-array
004	substrate
008	cell binding location
009	chemical modification of the cell binding locations
010	cells arrayed on the cell binding locations
012	chamber
013	depression formed at fluidic location
014	input channel
016	output channel
018	cassette
020	spacer supports
024	micro-capillary tubes
028	raised reservoir
030	first channel
032	second channel
036	plug
040	array of fluidic locations
044	luminescence reader instrument
048	first storage compartment
050	first robotic arm
052	
	computer
060	Optics
064	computer controlled x-y-z stage

068	computer-controlled rotating nosepiece
070	low-magnification objective
072	high-magnification objective
074	while-light source lamp
076	excitation filter wheel
078	dichroic filter system
080	emission filters
082	detector (e.g., cooled CCD)
086	computer screen
090	database
140	lateral space between wells
160	horizontal connecting channels
180	vertical connecting channels
200	switchable positive pressure source reservoirs or pumps
220	source reservoirs at atmospheric pressure
240	m x 1 valve-less manifold
300	waste reservoirs
320	switchable negative pressure waste reservoirs or pumps
340	thermodynamically-pumped waste reservoir
400	1 x n valve manifold
410	n x 1 valve manifold
420	2n x 1 valve manifold
500	Check Valve
520	Valve Element
540	Valve Seat
560	magnetic (B) field gradient

| 620 | Magnetic Field |

700	Fill ports
710	Channels
720	Valves
730	Reservoirs
740	Electrokinetic pump
760	Electrode 1
780	Electrode 2

Detailed Description Of The Preferred Embodiments

5   All patents, patent applications, and references cited herein are incorporated by reference in their entirety.

As used herein the term "well" is defined as the volume space created by the mating of one fluidic location of the chamber with one cell binding location on the substrate. The volume space may be created by a depression on the chamber corresponding to the fluidic location, by a depression on the substrate corresponding to the cell binding location, by a

10   spacer support that separates the chamber and the substrate (wherein there is no requirement for a depression in the substrate of the chamber), a combination of any of these, or any other suitable method for creating a volume space between the fluidic location and the cell binding location.

As used herein, the term "cell binding location" refers to a discrete location on the

15   substrate that comprises a plurality of cell binding sites capable of binding cells. The substrate may be derivatized to create cell binding sites, or the cell binding sites may naturally be present on the substrate. The plurality of cell binding sites within an individual cell binding location may be capable of binding only a single cell type, or may be capable of binding more than one cell type. Different cell binding locations on the same substrate may

20   be identical or different in the type of cells that they are capable of binding.

The term "fluidic location" as used herein refers to a discrete location on the chamber that is the site of fluid delivery to and/or removal from the cell binding location. The fluidic

an input and/or output channel or the terminus of a via that is in fluid connection with an input and/or output channel.

As used herein, the term "cassette" means the combination of the substrate and the chamber. The combination can be either modular (more than one piece), providing a re-usable chamber and a disposable substrate, or non-modular (single piece), to limit any potential interwell leaking.

5   As used herein, the term "on-board" means integral with the cassette.

As used herein, the term "integrated fluidics" means that a microfluidic device comprises both a substrate surface for cell binding and a chamber.

As used herein, the term "chamber" means a fluid delivery system comprising microfluidic channels and fluidic locations, which serves as a specialized substrate cover.

10   As used herein, the term "switchable pump" refers to a pump, including but not limited to a syringe pump, a pressure-controlled vessel with a valve, or an electrokinetic pump that is employed externally (with the optional use of electrical shielding) with respect to the matrix of the array of wells, so that no harmful electric field effects are experienced by the cells.

15   As used herein, the term "sub-array" means any contiguous sub-section of wells in the entire array of wells, typically in a format corresponding to the shape of an imaging detector array, such as a CCD (charge coupled device detector).

As used herein the term "matrix of the cell array" means the space defined by the imaginary parallelpiped (i.e., typically a cubic or rectangular box) that would fit around the 20 entire set of wells in the array of the cassette.

As used herein, the term "assay components" refers to any component that would be added to a cell screening assay, including but not limited to reagents, cells, test compounds, media, antibodies, luminescent and reporters.

As used herein, the term "luminescent" encompasses any type of light emission, 25 including but not limited to luminescence, fluorescence, and chemiluminescence.

In one aspect, the present invention teaches a method of making a micro-array of multiple cell types on a common substrate. As defined herein, a micro-array of multiple cell types refers to an array of cells on a substrate that are not distributed in a single uniform

binding selectivity.

The method of making a micro-array of multiple cell types comprises preparing a micro-patterned chemical array (also referred to herein as a chemically modified array of cell binding locations), chemically modifying the array non-uniformly, and binding cells to the non-uniform chemical array.

5   In a preferred embodiment, a micro-patterned chemical array comprises a substrate **4** which is treated to produce a hydrophobic surface across which are dispersed at regular intervals hydrophilic spots or "cell binding locations" **8**. (**Figure 1A-1B**). The substrate can be a glass, plastic, or silicon wafer, such as a conventional light microscope coverslip, but can also be made of any other suitable material to provide a substrate. As describe 10   previously, the term " cell binding locations" is used to describe a specific spot on the substrate, and does not require any particular depth. The surface of the substrate **4** is preferably about 2 cm by 3 cm, but can be larger or smaller. In a preferred embodiment, the cell binding locations **8** of the micro-patterned chemical array contain reactable functional groups such as, but not limited to, amino hydroxyl, sulfhydryl or carboxyl groups that can 15   bind to cells non-specifically or be further chemically modified to bind molecules that bind cells specifically.

Chemically modified cell binding locations are produced by specific chemical modifications of the cell binding locations on the substrate. The cell binding locations may comprise a variety of different cell binding molecules that permit attachment and growth of 20   different types of cells in the cell binding locations, or may permit attachment of only a single cell type. The hydrophobic domains surrounding the cell binding locations on the substrate do not support the attachment and growth of the cells.

In one embodiment, a micro-array of multiple cell types is made by coating a glass wafer via chemisorbance with a layer of a substance having reactable functional groups such 25   as amino groups. In a preferred embodiment, an aminosilane such as 3-amino propyltrimethoxysilane (APTS) or N-(2-aminoethyl-3-aminopropyl)trimethoxysilane (EDA) is used, but other reactable substances may be used. Following this first step, due to the presence of the reactable functional groups, the entire surface of the coated glass wafer is

substance having photo-cleavable or chemically removable amino protecting groups are placed in a micro-pattern of discrete locations on the aminosilane coated glass wafer. In one

embodiment the pattern comprises a rectangular or square array, but any suitable discrete pattern, may be used (such as, but not limited to, triangular or circular). In one embodiment, the drops range in volume from 1 nanoliter (nl) to 1000 nl. In a preferred embodiment the drops range from 250-500 nl in volume. Suitable photochemically removable amino protecting substances include, but are not limited to 4-bromomethyl-3-nitrobenzene, 1-(4,5-dimethoxy-2-nitrophenyl)-ethyl (DMNPE) and butyloxycarbonyl. In one embodiment, the patterning reaction is carried out for 1 to 100 minutes at temperatures ranging from ambient temperature to 37°C, using reagent concentrations of between 1 micromolar (uM) and 1000 uM. In a preferred embodiment, the reaction is carried out at 37°C for 60 minutes using a reagent concentration of 500 uM.

The drops may be placed onto the aminosilane coated glass wafer via conventional ink-jet technology. (U.S Patent No. 5,233,369; U.S. Patent No. 5,486,855). Alternatively, an array of pins, defined herein as tapered rods that can transfer between 1 nl and 1000 nl of fluid, is dipped into a bath of the amino protecting substance to produce drops of the protecting substance on their ends. The pins are then contacted with the glass wafer to transfer the drops thereto. In another embodiment, an array of capillary tubes made of glass or plastic, as described in U.S Patent Nos. 5,567,294 and 5,527,673, (both herein incorporated by reference), containing the amino protecting substance is contacted with the glass wafer to transfer the droplets to the surface. Thus, the glass wafer is micro-patterned with an array of spots or cell binding locations that contain protected amino groups on a hydrophobic surface (**Figure 2A-B**).

Third, a hydrophobic substance reactive with unprotected amino groups is washed over the glass wafer. The hydrophobic substance can be a fatty acid or an alkyl iodide, or any other suitable structure. Certain conditions for such a derivatization of glass can be found in Prime and Whitesides, Science 252:1164-1167, 1991, Lopez et al., J. Am. Chem. Soc. 115:5877-5878, 1993, and Mrksich and Whitesides, Ann. Rev. Biophys. Biomol. Struct. 25:55-78, 1996. The fatty acid or alkyl iodide reacts with the unprotected amino groups and covalently attaches thereto, and the amino groups are now hydrophobic due to the fatty acid

Fourth, the non-uniform array of cell binding locations is produced by uniformly de-protecting the amino groups in a micro-patterned chemical array produced according to the above-described methods. In one embodiment, chemical specificity can be added by chemically crosslinking specific molecules to the cell binding locations. There are a number of well known homo- or hetero-bi-functional crosslinking reagents such as ethylene glycol bis(succinimidylsuccinate) that will react with the free amino groups in the cell binding locations and crosslink to a specific molecule. Reagents and conditions for crosslinking free amino groups with other biomolecules are well known in the art, as exemplified by the following references: Grabarek and Gergely, Analyt. Biochem 185:131-135, 1990; McKenzie et al., J. Prot. Chem. 7:581-592, 1988; Brinkley, Bioconjugate Chem. 3:12-13; 1992, Fritsch et al., Bioconjugate Chem. 7:180-186, 1996; and Aplin and Hughes, 1981.

In a preferred embodiment, a non-uniform array of cell binding locations is produced in combinatorial fashion. The resulting cell binding locations are non-uniform (*i.e.*, each cell binding location or group of cell binding locations may be unique in its cell binding selectivity). By the term combinatorial, it is meant that the cell binding locations are variably treated.

In one embodiment, the protected amino groups of the modified array of cell binding locations of step 3 are de-protected and then specific molecules with chemical crosslinking reagents are deposited in a desired pattern. The specific crosslinking agents can bind to the amino groups and further possess a cell-binding group. In this step, the type of cell binding group can be varied from one cell binding location to another, or from one group of cell binding locations to another, to create a non-uniform design in the array.

In another embodiment, the amino groups of the chemically modified cell binding locations of step 3 are uniformly de-protected. A photo-activatable crosslinker is reacted with the de-protected amino groups. An optical mask of a desired pattern is placed over the surface of the cell binding locations and the exposed cell binding locations are illuminated with a light source. The position and number of cell binding locations that receive light is controlled by the micro-pattern of the optical mask. Suitable photo-activatable crosslinkers

al., Sigal et al., 1994, Sigal et al., 1996 and Mrksich and Whitesides, 1996. The photo-activatable crosslinker is bi-functional in that it chemically bonds to the amino group on the

cell binding locations and, when exposed to light, covalently bonds to cell binding molecules, such as antibodies. Reagents and conditions for photoactivated crosslinking are discussed in Thevenin et al., Eur. J. Biochem. 206:471-477, 1992 and Goldmacher et al., Bioconjugate Chem. 3:104-107, 1992.

5   When a photo-activatable crosslinker is used, the glass plate is flooded with cell binding molecules to be bound to the cell binding locations. In one embodiment, cell binding molecules such as cell surface antigen-reactive antibodies, extracellular matrix proteins, (for example, fibronectin or collagen) or charged polymers (for example poly-L-lysine or poly-L-arginine) are used in concentrations ranging from about 0.1 to about 1 mM. While the cell

10   binding molecules cover the cell binding locations, the glass plate is irradiated from the underside of the glass plate, at an angle below the critical angle of the material of the glass plate, resulting in total internal reflection of the light. (For discussion of total internal reflection fluorescence microscopy, see Thompson et al., 1993). In one embodiment, the irradiation is carried out at between ambient temperature and 37°C for 0.1 to 10 seconds with

15   light of wavelength between 300 nanometers (nm) to 1000 nm. In a preferred embodiment, the irradiation is conducted at ambient temperature for 1 second using light with a wavelength of between about 300 and 400 nm. Optical crosslinking limits the photo-activatable crosslinking to a short distance into the solution above the cell binding locations, and is described in Bailey et al., Nature 366:44-48, 1993; Farkas et al., Ann. Rev. Physiol.

20   55:785-817, 1993; Taylor et al., Soc. Opt. Instr. Eng. 2678:15-27, 1996; Thompson et al., in Mason, W.T. (ed.), "Fluorescent and Luminescent Probes for Biological Activity." San Diego: Academic Press pp. 405-419, 1993.

        The photo-activatable crosslinker binds with the cell binding molecules such as antibodies and matrix proteins, only in the cell binding locations where the crosslinker was

25   irradiated. For example, a single row of an array of cell binding locations can be irradiated to produce a single row of cell binding locations with cell binding molecules bound to the crosslinker. Following a washing of the array to eliminate any unbound cell binding molecule, a second row of cell binding locations can be bound to a second cell binding

molecules are removed by washing the array with PBS, or any other suitable buffer. In this fashion, multiple rows of cell binding locations or groups of cell binding locations can be

sequentially illuminated by sequential masking in the presence of a particular cell binding molecule. Alternatively, each cell binding location can be irradiated one by one using pinpoint exposure and optical masking. In this manner, different cell binding molecules are bound to rows of the array or to individual cell binding locations, creating a non-uniform array of cells bound to the cell binding locations of any desired pattern.

In a further embodiment for producing chemically modified arrays of cell binding locations, a chemically modified array is first produced wherein the amino groups of the cell binding locations are uniformly protected with photo-cleavable protecting groups. Rows, columns, and/or individual cell binding locations are sequentially photo-deprotected to expose the free amino groups by using an optical mask of various patterns to cover all but the cell binding locations to be de-protected. The exposed cell binding locations (i.e., those not covered by the mask), are illuminated, resulting in removal of the protecting groups. The array is flooded with a bi-functional crosslinker which chemically bonds to the de-protected amino group and activates the cell binding locations. Conditions for the photode-protection of amino groups are discussed in Padwa, A. (ed.) "Organic Photochemistry.", New York 9:225-323, 1987, Ten et al., Makromol. Chem. 190:69-82, 1989, Pillai, Synthesis 1980:1-26, 1980, Self and Thompson, Nature Medicine 2:817-820, 1996 and Senter et al., Photochem. Photobiol. 42:231-237, 1985. Next, cell binding molecules are flooded onto the modified chemical array wherein they react with the other half of the crosslinker. The array is then washed to eliminate any unbound bi-functional crosslinker and cell binding molecules. Another cell binding location or set of cell binding locations may be de-protected using another optical mask, and the array may then be flooded with a second treatment of a bi-functional crosslinker followed by a distinct cell binding molecule which bonds to this second cell binding location or set of cell binding location of de-protected amino groups. The array is washed to eliminate the second treatment of a bi-functional crosslinker and cell binding molecules. A non-uniform array of cell binding molecules may thus be produced by a repeated sequence of photo-de-protection, chemical crosslinking of specific molecules and washing under a variety of masks. Alternatively, the crosslinking reagents can be delivered

number of de-protected amino groups exposed using an optical mask, or by controlling the dose of irradiation for the photo-activatable crosslinkers.

The chemically modified array of cell binding locations is then used to produce a non-uniform array of cells on the cell binding locations. In one embodiment, the modified chemical array is "seeded" with cells by introducing suspended cells onto the array, allowing binding of the cells to the cell binding locations and then rinsing the wafer to remove

5   unbound and weakly bound cells. The cells are bound only in the cell binding locations, because the specific chemical environment in the cell binding locations, in conjunction with the hydrophobic environment surrounding each of the cell binding locations, permits the selective binding of cells to the cell binding locations only. Furthermore, the modification of cell binding locations with specific cell-binding molecules permits selective binding of cells

10   to specific cell binding locations, producing a non-uniform array of cells on the cell binding locations. In addition, the cell surface molecules that specifically bind to the cell binding locations may be either naturally present or genetically engineered by expressing "cell binding location-specific" molecules that have been fused to cellular trans-membrane molecules such that cells interact with and bind specifically to modified cell binding

15   locations. The creation of an array of cell binding locations with different cell recognition molecules allows one cell binding location, a group of cell binding locations, or the entire array to specifically "recognize", grow and screen cells from a mixed population of cells.

In one embodiment, cells suspended in culture medium at concentrations ranging from about 10^3 to about 10^7 cells per ml are incubated in contact with the cell binding

20   locations for 1 to 120 minutes at temperatures ranging from ambient temperature to 37^0C. Unbound cells are then rinsed off of the cell binding locations using culture medium or a high density solution to lift the unbound cells away from the bound cells. (Channavajjala, et al., J. Cell Sci. 110:249-256, 1997). In a preferred embodiment, cells suspended in culture medium at concentrations ranging from about 10^5 to about 10^6 cells per ml are incubated in contact

25   with the cell binding locations at 37°C for times ranging from about 10 minutes to about 2 hours.

The density of cells attached to the cell binding locations is controlled by the cell density in the cell suspension, the time permitted for cell attachment to the chemically

... are incubated at between ambient temperature and 37 C for between 1 minute and 120 minutes, with cell binding locations containing between 0.1 and 100 nmoles per cm^2 of cell

binding molecules. In a preferred embodiment, 10^5 and 10^6 cells per ml are incubated for 10 minutes to 2 hours at about 37°C, with cell binding locations containing about 10 to 100 nmoles per cm^2 of cell binding molecules.

In one embodiment, the cells may be chemically fixed to the cell binding locations as described by Bell et al., J. Histochem. Cytochem 35:1375-1380, 1987; Poot et al., J. Histochem. Cytochem 44:1363-1372, 1996; Johnson, J. Elect. Micros. Tech. 2:129-138, 1985, and then used for screening at a later time with luminescently labeled molecules such as antibodies, nucleic acid hybridization probes or other ligands.

In another embodiment, the cells can be modified with luminescent indicators of cell chemical or molecular properties, seeded onto the non-uniform chemically modified array of cell binding locations and analyzed in the living state. Examples of such indicators are provided in Giuilano et al., Ann. Rev. Biophys. Biomol. Struct. 24:405-434, 1995; Harootunian et al., Mol. Biol. Cell 4:993-1002, 1993; Post et al., Mol. Biol. Cell 6:1755-1768, 1995; Gonzalez and Tsien, Biophys. J. 69:1272-1280, 1995; Swaminathan et al., Biophys. J. 72:1900-1907, 1997 and Chalfie et al., Science 263:802-805, 1994 . The indicators can be introduced into the cells before or after they are seeded onto the array by any one or a combination of variety of physical methods, such as, but not limited to diffusion across the cell membrane (reviewed in Haugland, Handbook of fluorescent probes and research chemicals, 6th ed. Molecular Probes, Inc., Eugene, 1996), mechanical perturbation of the cell membrane (McNeil et al., J. Cell Biology 98:1556-1564, 1984; Clarke and McNeil, J. Cell Science 102:533-541, 1992; Clarke et al., BioTechniques 17:1118-1125, 1994), or genetic engineering so that they are expressed in cells under prescribed conditions. (Chalfie et al., 1994). In a preferred embodiment, the cells contain luminescent reporter genes, although other types of reporter genes, including those encoding chemiluminescent proteins, are also suitable. Live cell studies permit analysis of the physiological state of the cell as reported by luminescence during its life cycle or when contacted with a drug or other reactive substance.

In another aspect of the present invention, a non-uniform cell array on the cell binding

non-uniform, chemically modified array of cell binding locations provides a variety of cell binding sites of different specificity. Any cell type can be arrayed, providing that a molecule

capable of specifically binding that cell type is present in the chemically modified array of cell binding locations. Preferred cell types for the non-uniform cell array on the cell binding locations include lymphocytes, cancer cells, neurons, fungi, bacteria and other prokaryotic and eukaryotic organisms. For example, **Figure 3A** shows a non-uniform cell array on the

5   cell binding locations containing fibroblastic cells grown on a surface patterned substrate and labeled with two fluorescent probes (rhodamine to stain actin and Hoechst to stain nuclei), while **Figure 3B** shows a non-uniform cell array on the cell binding locations containing fibroblastic cell growth (L929 and 3T3 cells) in spotted patterns, labeled with two fluorescent probes and visualized at different magnifications.

10   Examples of cell-binding molecules that can be used in the non-uniform cell array on the cell binding locations include, but are not limited to antibodies, lectins and extracellular matrix proteins. Alternatively, genetically engineered cells that express specific cell surface markers can selectively bind directly to the modified cell binding locations. The non-uniform cell array on the cell binding locations may comprise either fixed or living cells. In a

15   preferred embodiment, the non-uniform cell array on the cell binding locations comprises living cells such as, but not limited to, cells "labeled" with luminescent indicators of cell chemical or molecular properties.

             In another aspect of the present invention, a method for analyzing cells is provided, comprising preparing a non-uniform cell array on the cell binding locations wherein the cells

20   contain at least one luminescent reporter molecule, contacting the non-uniform cell array on the cell binding locations to a fluid delivery system to enable reagent delivery to the cells, conducting high-throughput screening by acquiring luminescence image of the entire non-uniform cell array at low magnification to detect luminescence signals from all cell binding locations at once to identify those that exhibit a response. This is followed by high-content

25   detection within the responding cell binding locations using a set of luminescent reagents with different physiological and spectral properties, scanning the selected cell binding locations to obtain luminescence signals from the luminescent reporter molecules in the cells, converting the luminescence signals into digital data and utilizing the digital data to

 preferred embodiments of the non-uniform cell array on the cell binding locations are disclosed above. In a preferred embodiment of the fluid delivery system, a chamber, mates

with the substrate containing the non-uniform cell array. The chamber is preferably made of glass, plastic or silicon, but any other material that can provide a chamber is suitable. One embodiment of the chamber **12** shown in **Figure 4** has an array of etched domains **13** matching the cell binding locations **8** on the substrate **4**. In addition, input channels **14** are

5   etched to supply fluid to the etched domains **13**. A series of "output" channels **16**, to remove excess fluid from the etched domains **13**, can also be connected to the cell binding locations. The chamber **12** and substrate **10** together constitute a cassette **18**. While this embodiment utilizes etched domains, any other type of depression **13** formed at the fluidic location **1** can also be utilized in this embodiment. Alternatively, the fluidic location may be flat and the

10 cell binding locations **8** may comprise depressions that match the fluidic locations **1**. In another alternative, both the cell binding site **8** and the fluidic location **1** are flat, and a volume space for the well is created by the use of a spacer support **20** between the substrate **4** and the chamber **12**.

The chamber **12** is thus used for delivery of fluid to the cells arrayed on the cell

15 binding locations **10**. The fluid can include, but is not limited to a solution of a particular drug, protein, ligand, or other substance which binds with surface expressed moieties of cells or that are taken up by the cells. The fluid to interact with the cells arrayed on the cell binding locations **10** can also include liposomes encapsulating a drug. In one embodiment, such a liposome is formed from a photochromic material, which releases the drug upon

20 exposure to light, such as photoresponsive synthetic polymers. (Reviewed in Willner and Rubin, Chem. Int. Ed. Engl. 35:367-385, 1996). The drug can be released from the liposomes in all channels **14** simultaneously, or individual channels or separate rows of channels may be illuminated to release the drug sequentially. Such controlled release of the drug may be used in kinetic studies and live cell studies. Control of fluid delivery can be accomplished by a

25 combination of micro-valves and micro-pumps that are well known in the capillary action art. (United States Patent No. 5,567,294; United States Patent No. 5,527,673; United States Patent No. 5,585,069, all herein incorporated by reference.)

Another embodiment of the chamber **12** shown in **Figure 5** has an array of input

[illegible lines]

the etched domains **13** of the chamber **12**. Spacer supports **20** are placed between the chamber **12** and the cells arrayed on the cell binding locations **10** along the sides of contact.

The substrate 4 and the chamber 12 can be sealed together using an elastomer or other sticky coating on the raised region of the chamber. Each etched domain 13 of the chamber 12 can be individually or uniformly filled with a medium that supports the growth and/or health of the cells arrayed on the cell binding locations 10. In a further embodiment (**Figure 6**), the chamber contains no input channels, for treating all the cells arrayed on the cell binding locations 10 with the same solution.

Delivery of drugs or other substances is accomplished by use of various modifications of the chamber as follows. A solution of the drug to be tested for interaction with cells of the array can be loaded from a 96 well microtiter plate into an array of micro-capillary tubes 24. (**Figure 7**). The array of micro-capillary tubes 24 corresponds one-to-one with the input channels 14 of the chamber 12, allowing solution to flow or be pumped out of the micro-capillary tubes 24 into the channels 14. The cassette 18 is inverted so that the cell binding locations 8 become submerged in the etched domain 13 filled with the fluid (**Figure 7B**). Once the interaction between the fluid and cells occurs, luminescence signals emanating from the cells arrayed on the cell binding locations 10 can be measured directly or, alternatively, the substrate 4 can be lifted off the chamber for post processing, fixation, and labeling. The placement and removal of the array of cells may be accomplished via robotics and/or hydraulic mechanisms. (Schroeder and Neagle, 1996)

In one embodiment of the chamber 12 shown in **Figure 7,** the channels and matching etched domains 13 are etched into the chamber chemically (Prime and Whitesides, 1991; Lopez et al., 1993; Mrksich and Whitesides, 1996). The etched domains 13 are larger in diameter than the cell binding locations 8. This permits the chamber 12 to be contact sealed to the substrate 4, leaving space for the cells and a small volume of fluid. Input channels 14 are etched into each row of etched domains 13 of the chamber 12. Each input channel 14 extends from two opposing edges of the chamber 12 and is open at each edge. The etched domains 13 of a single row are in fluid communication with the input channels 14 by placing a micro-capillary tube 24 containing a solution into contact with the edge of the chamber 12. Each row of connected input channels 14 can be filled simultaneously or sequentially. During filling of the

In a further embodiment of the chamber **12**, raised reservoirs **28** and input channels **14** can be placed onto the surface of the chamber **12** as shown in **Figure 8b**. In a preferred embodiment, the raised reservoirs **28** and input channels **14** can be made from polytetrafluoroethylene or elastomeric material, but they can be made from any other sticky material that permits attachment to the substrate **4**, such as poly(dimethylsiloxane), manufacture by Dow Corning under the trade name SYLGARD 184™. The effect is the same as with a chamber having etched channels and channels and its uses are similar.

In another embodiment of the chamber shown in **Figure 8A**, a first channel **30** extends from one edge of the chamber **12** to a first etched domain **13** or raised reservoirs **28** and channels. A second channel **32** extends from the opposing edge to a second etched domain adjacent the first etched domain. The first **30** and second **32** channels are not in fluid communication with each other yet are in the same row of input channels **14** or raised reservoirs **28**.

In another embodiment, as shown in **Figures 9 and 10**, the chamber **12** may have an input channel **14** extending from each etched domain **13** or raised reservoir **28** to the edge of the chamber. The channels **14** can all originate from one edge of the chamber **12** (**Figure 9**), or from both edges (**Figure 10**). The input channels **14** can also be split to both sides of the etched domains **13** to minimize the space occupied by the input channels **14**. Separate fluidic channels allow for performance of kinetic studies where one row at a time or one depression at a time is charged with the drug.

In a further embodiment depicted in **Figure 11**, each etched domain **13** is in fluid communication with a corresponding input channel **14** having a plug **36** between the end of the channel **14** and the etched domain **13**, which prevents the injected solution from flowing into the etched domain **13** until the desired time. Solutions may be preloaded into the input channels **14** for use at a later time. A plug **36** likewise can be disposed between a terminal etched domain **13** in a set of connected etched domains **13** in fluid communication with an input channel **14**. Upon release of the plug **36**, the substance flows through and fills all the etched domains **13** which are in fluid communication with the input channel **14**.

...dable crosslinkers that, upon irradiation, becomes hydrophilic and passes along with the drug into the depression. Alternatively, the plug **36** may be formed of a crosslinked polymer,

such as proteins, carbohydrates or lipids that have been crosslinked with photo-cleavable crosslinkers that, when irradiated, decomposes and passes into the etched domain 13 along with the solution.

The cassette 18, which comprises the substrate 4 and the chamber 12, is inserted into a luminescence reader instrument. The luminescence reader instrument is an optical-mechanical device that handles the cassette, controls the environment, controls delivery of solutions to wells, and analyzes the luminescence emitted from the array of cells, either one well at a time or the whole array simultaneously. In a preferred embodiment (Figure 12), the luminescence reader instrument 44 comprises an integrated circuit inspection station using a fluorescence microscope as the reader and microrobotics to manipulate the cassettes. The reader of the present invention can comprise any optical system designed to image a luminescent specimen onto a detector. A storage compartment 48 holds the cassettes 18, from where they are retrieved by a robotic arm 50 that is controlled by computer 56. The robotic arm 50 inserts the cassette 18 into the luminescence reader instrument 44. The cassette 18 is removed from the luminescence reader instrument 44 by another robotic arm 52, which places the cassette 18 into a second storage compartment 54.

The luminescence reader instrument 44 is an optical-mechanical device designed as a modification of light optical-based, integrated circuit inspection stations used to "screen" integrated circuit "chips" for defects. Systems integrating environmental control, micro-robotics and optical readers are produced by companies such as Carl Zeiss [Jena, GmbH]. In addition to facilitating robotic handling, fluid delivery, and fast and precise scanning, two reading modes, high content and high throughput are supported. High-content readout is essentially the same as that performed by the ARRAYSCAN™ reader (U.S. Patent No. 5,989,835). In the high content mode, each location on the micro-array of multiple cell types is imaged at magnifications of 5-40X or more, recording a sufficient number of fields to achieve the desired statistical resolution of the measurement(s).

In the high throughput mode, the luminescence reader instrument 44 images the micro-array of multiple cell types at a much lower magnification of 0.2X to 1.0X magnification. ...

... multiple cell types imaged at ... X magnification would fill a 1000 x 1500 array of 10um pixels, yielding 20um/pixel resolution, insufficient to define intracellular

luminescence distributions, but sufficient to record an average response in a single well, and to count the numbers of a particular cell subtype in a well. Since typical integration times are on the order of seconds, the high throughput mode of reading technology, coupled with automated loading and handling, allows for the screening hundreds of compounds a minute.

5   In one embodiment shown in **Figure 13**, the luminescence reader instrument comprises an optical-mechanical design that is either an upright or inverted fluorescence microscope **44**, which comprises a computer-controlled x,y,z-stage **64**, a computer-controlled rotating nosepiece **68** holding a low magnification objective **70** (*e.g.*, 0.5 x) and one or more higher magnification objectives **72**, a white light source lamp **74** with excitation filter wheel

10   **76**, a dichroic filter system **78** with emission filters **80**, and a detector **82** (*e.g.*, cooled charge-coupled device). For the high throughput mode, the low magnification objective **70** is moved into place and one or more luminescence images of the entire cell array is recorded. Wells that exhibit some selected luminescence response are identified and further analyzed via high content screening, wherein the nosepiece **68** is rotated to select a higher magnification

15   objective **72** and the x,y,z-stage **64** is adjusted to center the "selected" well for both cellular and subcellular high content screening, as described in U.S. Patent No. 5,989,835.

In an alternate embodiment, the luminescence reader instrument **44** can utilize a scanned laser beam in either confocal or standard illumination mode. Spectral selection is based on multiple laser lines or a group of separate laser diodes, as manufactured by Carl

20   Zeiss (Jena, GmbH, Germany) or as discussed in Denk, et al. (Science 248:73, 1990).

Another embodiment of the high throughput screening mode involves the use of a low-resolution system consisting of an array (1 x 8, 1 x 12, etc.) of luminescence exciters and luminescence emission detectors that scans subsets of the wells on a non-uniform micro-patterned array of cells. In a preferred embodiment, this system consists of bundled optical

25   fibers, but any system that directs luminescence excitation light and collects luminescence emission light from the same well will suffice. Scanning the entire micro-array of multiple cell types with this system yields the total luminescence from each well, both from cells and the solution they are bathed in. This embodiment allows for the collection of luminescence

multiple cell types in both the high throughput and high content modes using the luminescence reader instrument, which first uses high throughput detection to measure a

response from the entire array "A". (**Figure 14B**). Any well that responds above a preset threshold is considered a hit and the cells in that well are measured via high content screening. (**Figure 14C**). The high content mode ("B") may or may not measure the same cell parameter measured during the high throughput mode ("A").

5   In another aspect of the invention, a cell screening system is disclosed, wherein the term "screening system" comprises the integration of a luminescence reader instrument, a cassette that can be inserted into the luminescent reader instrument comprising a micro-array of multiple cell types wherein the cells contain at least one luminescent reporter molecule and a chamber associated with the non-uniform micro-patterned array of cells, a digital detector

10   for receiving data from the luminescence reader instrument, and a computer means for receiving and processing digital data from the digital detector.

Preferred embodiments of the luminescence reader instrument, and the cassette comprising the micro-array of multiple cell types and the chamber are disclosed above. A preferred embodiment of the digital detector is disclosed in U.S. Patent No. 5,989,835, and

15   comprises a high resolution digital camera that acquires luminescence data from the luminescence reader instrument and converts it to digital data. In a preferred embodiment, the computer means comprises a digital cable that transports the digital signals from the digital detector to the computer, a display for user interaction and display of assay results, a means for processing assay results, and a digital storage media for data storage and archiving,

20   as described in U.S. Patent No. 5,989,835.

In a preferred embodiment, the cell screening system of the present invention comprises integration of the preferred embodiments of the elements disclosed above (**Figure 15**). The array of multiple cell types **10** comprises cells bound to chemically modified cell binding locations **8** on a substrate **4**. The chamber **12** serves as a microfluidic delivery

25   system for the addition of compounds to the cells **10** on the substrate **4**, and the combination of the two comprises the cassette **18**. The cassette **18** is placed in a luminescence reader instrument **44**. Digital data are processed as described above and in U.S. Patent No. 5,989,835, hereby incorporated by reference in its entirety. The data can be displayed on a computer screen 86 and m

relating to previous experiments with the cells. An example of the computer display screen is shown in **Figure 16.**

Example 1.   Coupling of antibodies to micro-array of multiple cell types for the attachment
of specific lymphoid cells

1. The cell line used was a mouse B cell lymphoma line (A20) that does not express IgM on its surface. A micro-array of multiple cell types was prepared for derivatization by being immersed overnight in 20% sulfuric acid, washed 2-3 times in excess distilled water, rinsed in 0.1M sodium hydroxide and blotted dry. The micro-array of multiple cell types was either used immediately or placed in a clean glass beaker and covered with parafilm for future use.

2. The micro-array of multiple cell types was placed in a 60mm petri dish, and 3-Aminopropyltrimethoxysilane was layered onto the micro-array of multiple cell types ensuring complete coverage without running over the edges (approximately 0.2ml for a 22 x 22 mm non-uniform micro-patterned array of cells, and approximately 0.5ml for a 22 x 40 mm non-uniform micro-patterned array of cells). After 4 minutes at room temperature, the micro-array of multiple cell types was washed in deionized water and excess water was removed by blotting.

3. The micro-array of multiple cell types was placed in clean 60mm petri dishes and incubated with glutaraldehyde (2.5% in PBS, approximately 2.5ml) for 30 minutes at room temperature, followed by three PBS washes. Excess PBS was removed by blotting.

4. Cell nuclei in the micro-array of multiple cell types were labeled with a luminescent Hoechst dye during the blocking step. The appropriate number of lymphoid cells (see below) in C-DMEM were transferred to a 15 ml conical tube, and Hoechst dye was added to a final concentration of 10 μg/ml. Cells were incubated for 10-20 minutes at 37°C in 5% CO_2, and then pelleted by centrifugation at 1000 x *g* for 7 minutes at room temperature. The supernatant containing unbound Hoechst dye was removed and fresh media (C-DMEM) was added to resuspend the cells as follows: approximately 1.25 –1.5

5. The micro-array of multiple cell types was washed briefly in PBS and transferred to a clean, dry 60 mm petri dish, without touching the sides of the dish. Cells were carefully pipeted onto the top of the micro-array of multiple cell types at the density noted above. Dishes were incubated at 37°C in 5% CO_2 for 1 hour. Unbound cells were then removed by repeated PBS washings.

6. Antibody solutions (Goat Anti-Mouse IgM or Goat Anti-Mouse Whole Serum) were spotted onto parafilm (50 µl for 22 x 22 mm non-uniform micro-patterned array of cells, 100 µl for a 22 x 40 mm non-uniform micro-patterned array of cells). The micro-array of multiple cell types was inverted onto the spots, so that the antiserum covered the entire surface of the treated micro-array of multiple cell types without trapping air bubbles. The micro-array of multiple cell types was incubated with the antibody solution for 1 hour at room temperature.

7. The micro-array of multiple cell types was carefully lifted from the parafilm, placed in a clean 60 mm petri dish, and washed three times with PBS. Unreacted sites are then blocked by the addition of 2.5 ml of 10% serum (calf or fetal calf serum in DMEM or Hank's Balanced Salt Solution) for 1 hour at room temperature.

8. Both cell lines should bind to the anti-mouse whole serum, but only the X16s should bind to the anti-mouse IgM. The binding of specific lymphoid cell strains to the chemically modified surface is shown in **Figure 17**. The mouse lymphoid A20 cell line, lacking surface IgM molecules but displaying IgG molecules, bound much more strongly to the surface modified with whole goat anti-mouse serum (**Figure 17C**) than to the surface modified with goat anti-mouse IgM (**Figure 17B**) or an uncoated slide (**Figure 17A**).

Example 2.   High-Content and High Throughput Screen.

The insulin-dependent stimulation of glucose uptake into cells such as adipocytes and myocytes requires a complex orchestration of cytoplasmic processes that result in the translocation of GLUT4 glucose transporters from an intracellular compartment to the plasma membrane. A number of molecular events are triggered by insulin binding to its receptor, including [illegible] ...

... important role in cytoplasmic organization, intracellular signaling ions and molecules that regulate this living gel can also be considered as intermediates of GLUT4 translocation.

A two level screen for insulin mimetics is implemented as follows. Cells carrying a stable chimera of GLUT4 with a Blue Fluorescent Protein (BFP) are arranged on the micro-array of multiple cell types arrays, and then loaded with the acetoxymethylester form of Fluo-3, a calcium indicator (green fluorescence). The array of locations are then simultaneously

5   treated with an array of compounds using the microfluidic delivery system, and a short sequence of Fluo-3 images of the whole micro-array of multiple cell types are analyzed for wells exhibiting a calcium response in the high throughput mode. The wells containing compounds that induced a response, are then analyzed on a cell by cell basis for evidence of GLUT4 translocation to the plasma membrane (*i.e.*, the high-content mode) using blue

10   fluorescence detected in time and space.

Figure 18 depicts the sequential images of the whole micro-array of multiple cell types in the high throughput mode (**Figure 18A**) and the high content mode (**Figure 18B**). **Figure 19** shows the cell data from the high content mode.

15   *Example 3.   Improved Cassettes and Enhanced Well Density*

In another aspect, the present invention provides devices and methods for maximizing the cell-plated area and the number of wells that can be imaged in a sub-array, while still obtaining adequate pixel resolution in the image. This result has been achieved through the use of fluidic architectures that minimize the distance and area between wells, thus

20   maximizing well density.

In one embodiment of this aspect is provided a cassette for cell screening comprising a substrate having a surface, wherein the surface contains a plurality of cell binding locations; a fluid delivery system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the

25   substrate, wherein the multi-level chamber comprises

i.   a crossed array of microfluidic input channels and output channels, wherein each well is in fluid connection with one or more input channels and one or more output channels;

one or more input manifolds in fluid connection with the microfluidic input channels;

iv.   one or more output manifolds in fluid connection with the microfluidic output channels;

v.   at least one source receptacle in fluid connection with the one or more input manifolds; and

vi.   at least one waste receptacle in fluid connection with one or more output manifolds; and

a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location.

In preferred embodiments, the cassettes further comprise a pump to control fluid flow within the microfluidic device; a substrate temperature controller and/or a controller to regulate oxygen and carbon dioxide partial pressures within the device.

The substrate of the present invention is chemically modified so that cells selectively adhere to a cell growth substrate and are contained within small regions, referred to as "cell binding locations", which are sub-millimeter to a few millimeters in size. The combination of a cell binding location on the substrate and a fluidic location on the chamber defines a space referred to as a "well". The substrate may be predominantly flat and the cell binding locations may be matched with fluidic location depressions on a substrate cover, so that when the two parts are assembled there is some depth in the area of the wells so formed. Conversely, the substrate cover fluidic location may be flat and the substrate may contain depressions at the cell binding location, so that when the two parts are assembled there is some depth in the area of the wells so formed. Fluids and test compounds are supplied to the wells via a chamber, which combines with the substrate to form a cassette. In a preferred embodiment, the chamber acts as the substrate cover and contains fluidic locations in fluid connection with the microfluidic channels of the device, wherein the fluidic locations comprise depressions that match the cell binding locations on the substrate, such that the volume of each well is etched into the chamber.

On the surface of the substrate, there are sealing regions that lie between and around

chamber are joined, the sealing regions of both components meet to form a seal that prevents the flow of fluid across the sealing regions between the wells thereby formed. These sealing

regions of both components may be physically and/or chemically modified to enhance sealing. In a first example, a hydrophobic silane coating of octadecyltrichlorosilane is coated on the sealing regions. In a second example, light-curable adhesives and cyanoacrylates that are USP Class IV-compliant such as are available from Dymax Corporation are amenable to

5   use with biomedical devices made from ceramic, glass, plastic, or metal. In a third example, a biocompatible tape material with precision cut-outs that match the wells of the array is available from Avery Dennison Specialty Tape Division. Similarly, biomedical acrylic adhesives can be transferred from tapes to surfaces, such as are available from Tyco Corporation, for applying an adhesive coating precisely to these sealing regions. In a fourth

10   example, a light-curable silicone elastomer can be used for bonding and sealing between the sealing regions of the substrate and the chamber, such as are available from Master Bond, Inc.

      The microfluidic cassette of the present invention has advantages over prior art cell array microfluidic devices used in high content screening systems including, but not limited

15   to:

      1) faster imaging of a statistically-relevant set of cells (based on imaging a set of cell binding locations (i.e.: "sub-arrays") simultaneously; See **Fig. 20**)

      2) more wells per plate and less physical space occupied per plate (i.e.: higher well density),

      3) more efficient use of valuable test compounds, and

20   4) more efficient use of cells compared to microplates in which the entire well area is not imaged.

      As discussed *supra*, prior microwell plate fluid exchange for each well can only be achieved by means of a pipette being inserted into the well and either ejecting or aspirating

25   fluid to or from the well, respectively. Automated fluid handling in prior microwell plates is acheived by using robotic pipettors that insert a pipette into each well for each fluid transfer step in a procedure. This pipette is typically required also to move to or from another microplate well for the source or destination of the fluid that is exchanged with the microwell

      compressible, a pipette fluids with an acceptable degree of spatial resolution and accuracy. An acceptable level of accuracy requires that the measurement error

should not exceed 2%. Thus, for a 100 microliter (µl) volume in the well of a 96 well plate, a measurement error of 2 µl is acceptable. For a 100 nano-liter (nl) volume, a 2 nl measurement error would be required. This level of accuracy is not reliably available with prior automatic pipettors across a spectrum of compositions and viscosities.

5   The minimization of the inter-well distances enables the fast parallel reading of sub-arrays of wells. If the integrated fluidics is too bulky (i.e.: does not allow close-spacing of wells), then such fast parallel reading capability is lost. In a preferred embodiment of the present invention, a crossed channel architecture is utilized, allowing for fewer channels, valves, and pumps, and thereby further reducing the space taken by channels on the cassette

10   of the invention. In a most preferred embodiment, the channels are placed in levels above that of the wells, permitting the closest possible inter-well spacing. In a further preferred embodiment, the use of porous medium as a drain 'pump' allows for simpler design and fabrication of the system. According to the invention, the channels may be of any size that permits the fluidic architecture herein defined. In a preferred embodiment, they range in size

15   from between about 0.025 mm to about 0.5 mm in width for square cross-section channels, or in diameter for circular cross-section channels.

           The fluidic architecture of the instant microfluidic cassette enables the controlling elements (including, but not limited to pumps, manifolds, controlled-pressure vessels, and/or valves) to be located outside the matrix of the cell array. Figure 40 shows one embodiment

20   of the pumping and valving schemes of this invention as it would appear with all the pumps and valves on-board the cassette. All variations of the pumping and valving schemes shown in various Figures (and others that would be apparent to those skilled in the art) may be implemented in this way, either with on-board active devices outside the matrix of the wells, with off-board active devices, or with some devices on-board and some devices off-board.

25   As a result, the controlling elements can be either within the cassette (on-board) or external to the cassette (off-board). In either case, there are no active components within the matrix of the array of wells that might limit the close-spacing of channels and wells. Because this cassette is particularly designed for the culture and analysis of living cells, the fluidic

the functional parts that are designed for live cell culture are identified below.

In a preferred embodiment, the cassette of the present invention comprises an off-board pump to provide pressure-driven flow that also controls which wells are addressed by the flow. This design feature ensures that the pumping method, unlike many prior art methods, is not ineffective or harmful when used with cell culture medium that is aqueous,

5   polar and contains proteins and salts. For example, electrohydrodynamic pumping is ineffective with polar solvents (Marc Madou, Fundamentals of Microfabrication, CRC Press, Boca Raton, 1997, p. 433). Electro-osmosis is typically accompanied by some degree of electrophoretic separation of charged medium components, such as proteins. Also, electro-osmosis typically requires the use of electric field strengths within the range of 100 V/cm to

10 1000 V/cm, which may affect the physiology of the living cells in the device. The fluid control system of the present device does not suffer from the disadvantages of electrically-induced methods when using biological fluids. .

In another embodiment, the pump is on-board and may utilize electrically-driven flow, but the electric fields are limited to regions external to the matrix of the array of wells,

15 and no electric field gradients are applied across any of the wells in which live cells are present. This embodiment is shown in **Figure 41**, where two electrodes are used to apply electric potential differences along segments of fluidic channels corresponding to different on-board source reservoirs (730). Each channel segment with its electrode pair form an electrokinetic pump (740). These source reservoirs (730) may be supplied with media and/or

20 compounds via fill ports (700) in the cassette surface. Valves (720) between the reservoirs and the pumps are closed whenever the corresponding electrokinetic pump is off, and are open whenever the corresponding electrokinetic pump is on, to maintain back pressure and to allow intake of air or fluid, respectively. Control of the voltage applied to electrode 1 (760) is used to induce electrokinetic flow of the fluid in the corresponding channel segment.

25 Electrode 2 (780) (nearest to the matrix of wells) is kept at ground potential, as is the matrix of the wells, to ensure that minimal or no potential difference and/or electric field is developed in the vicinity of the cells, because electric fields are known to affect cell physiology. Alternatively, the electrokinetic pump may be located down stream from the

cells and ... ... way to the waste reservoir.

In all embodiments, the use of on-board or off-board pumps that are external to the matrix of wells to control fluid flow eliminates the need for active valves within the matrix of wells, and thus eliminates the problem of allocating space within the matrix that might prevent the close spacing of the wells.

The control of the fluid pathway by means of pressure control also enables the optional use of various diffusion control means that are compatible and effective with cell culture medium that is aqueous, polar and contains proteins and salts. These means also require minimal space on the device, and thus do not prevent the close-spacing of wells.

U.S. Patent No. 5,603,351 describes a microfluidic device that includes crossed channels, but the channel network is not defined to allow two or more reagents to be combined in a reaction well, but rather to allow reagents to be fed to a well in a serial fashion. The purpose of the present cassette is not to expose two or more different fluids to each other, but to expose the living cells cultured on the well bottoms in serial fashion to two or more different fluids.

The fluidic channels of the cassette of the present invention provide transport of fluid and compounds from source receptacles to each well and from each well to one or more waste receptacles. Each well could be provided with a separate input and output channel (**Fig. 21**), yielding $2n^2$ channels (100, 120) for an $n \times n$ array (040). To control the flow of m liquid or vapor mixtures to an $n \times n$ array, $m + n^2$ valves or pumps are required (**Fig. 22**). However, for a large array, the number of channels, pumps, and valves for this type of architecture becomes unwieldy. A pump that has variable or switchable pressure (e.g., a syringe pump) is functionally equivalent to a pressurized reservoir with a valve. Therefore, in the following discussions of pumps, reservoirs, and valves, it should be understood that these components may be substituted without altering the spirit of the invention.

Crossed-channel multilevel architecture

A preferred embodiment of architectures of the cassette consisting of a crossed, nonintersecting array of input (100) and output (120) channels and utilizing multiple levels is disclosed. Specif

because architectures of this class require fewer independent channels per well compared to the class of architectures with separate input and output channels for each well,

these crossed-channel architectures also minimize the lateral space (140) that is required between wells to accommodate the pathways for the channels.

The crossed-channel, multilevel architectures require only *2n* channels to address n^2 wells. In a preferred embodiment, the row and column channels themselves are formed in two distinct layers so that they do not intersect. The row channels lie above the column channels, or vice versa. These two layers lie either in the same plane of the well layer (**Figures 23 and 24**), or lie in a plane above the well layer (**Figs. 25-26** and **33-34**). In other embodiments, structures with more than two planes are contemplated.

In a most preferred embodiment, the channels are placed above the plane of the wells, so that the minimum inter-well distance (140) is constrained only by the optical and physical requirements for a certain wall thickness separating each well. The advantage of placing the channels in levels above the wells is shown by the following examples. Wells that are 0.4 mm x 0.4 mm can be placed on a 0.5 mm pitch grid with allowance for a 0.1 mm wall between wells, yielding a 5 mm x 5 mm area for a 10 x 10 well array. Row and column channels of 0.2 mm width can easily be formed in upper levels of the device with ample distance between the channels. By contrast, if 0.2 mm channels with 0.1 mm walls are required within the plane of the wells (and row and column channel layers are formed in two separate planes within the plane of the wells), the well pitch increases to 0.8 mm. This yields an 8 mm x 8 mm area for a 10 x 10 well array. In this example, the placement of the channels between the wells increases the area required for the array by a factor of 2.6. For different channel architectures with separate input and output channels for each well, the additional space required between wells is much greater.

Either for channels above or within the level of the wells, horizontal (160) or vertical (180) connecting channels or vias are formed from each well *(j,k)* to its corresponding row channel *j* and column channel *k*. Fabrication of such structures from glass, semiconductor, or plastic materials is well known in the art. In one such method, silicon dioxide (SiO_2) is lithographically formed within silicon nitride (Si_3N_4) layers using a multistep layer-by-layer process. (Turner and Craighead, Proc. SPIE 3528:114-117 (1998)) Exposure of the structure

Control of flow in the crossed-channel architecture

In a crossed array of rows *(A, B, C, etc.)* and columns *(1, 2, 3, etc.)* the flow of fluid to and from well *(j,k)* is produced when, for example, positive pressure is applied to row *j* and the valve of output channel *k* is opened, where *j* and *k* refer to, e.g., row *D* and column *7*,

5   respectively. Flow to all wells of an entire row *j* is produced when, for example, positive pressure is applied to row *j* and the valves of all output channels *(1, 2, 3, etc.)* are opened. A similar procedure would yield flow to all wells of a column.

The pressures that are discussed here are actually pressure differences relative to ambient atmospheric pressure, otherwise known as "gauge" pressure. Thus, the value of the

10 positive pressure is the amount by which an applied pressure is greater than atmospheric pressure; the value of the negative pressure is the amount by which an applied pressure is less than atmospheric pressure. These pressures are referred to as positive and negative gauge pressure, respectively.

The term "fluid pathway" is used herein indicates the particular set of wells {(j,k),

15 (j,n), (m,k), (m,n) … etc.} that is subjected to fluid flow by the enabling of the flow control devices of rows j and m, etc., and columns k and n, etc. Note that enabling the flow control devices of two rows and two columns yields a fluid pathway through four wells. This is defined herein as a single fluid pathway involving four wells, rather than four pathways involving single wells.

20

Reduction in the number of active valves or pumps

Various combinations of actively-controlled flow control devices (pumps or pressurized reservoirs with valves) are used to produce flow through a desired well (010) or fluid pathway. For example, *m* compounds can be multiplexed to a single well by using *m*

25 positive pressure source reservoirs or pumps (200), a valve-less manifold (240) and valve-free waste reservoir at atmospheric pressure (300)(**Fig. 27**). This valve-less manifold (240) can be replaced by a valved manifold [e.g., (260) of **Fig. 28**] to better control the diffusion of fluids between the *m* sources. Alternatively, the same functionality can be achieved by using

pressure allows convenient bubbling of gases through the source reservoirs to establish and maintain desired levels of dissolved CO_2 and O_2 in the media.

In another embodiment, a waste reservoir filled with a porous medium serves as a capillary action pump (340) and provides negative pressure (**Fig. 28**). The thermodynamics of wetting creates a pressure across a liquid-vapor interface bounded by a solid-liquid-vapor contact line. This pressure of capillary action can be used to draw a column of liquid from any microchannel pathway into the high surface area porous medium. A series of such negative pressure reservoirs can be linked to the array via a multiplexing valved manifold for control of flow, or one single reservoir can be closely coupled to the output channels of all wells simultaneously. A number of porous media are known in the art, including, but not limited to silica gel technology, porous ceramic materials such as zeolite, a pad of hydrophilic (synthetic or natural) fibers, and partially hydrated or lyophilized hydrogels such as alginates, poly(vinyl) alcohol gels, agarose, sugar polyacrylate gels, and polyacrylamide gels. In a preferred embodiment, the porous medium is based on silica gel technology. A thin membrane or filtering sheet may be used to partition the porous media from the microchannel array. This method can provide pressure for pumping until the internal surface area of the porous medium is largely wetted by the liquid. By suitable choice of porous medium and design of this method, adequately large amounts of liquid can be pumped to conduct a chosen measurement with the array system of the present invention.

In another embodiment, the array of m positive pressure source reservoirs or pumps (200) shown in **Fig. 27** is multiplexed to an $n \times n$ crossed channel array of wells (040) by means of $1 \times n$ input (400) and $n \times 1$ output (410) valve manifolds (**Fig. 29**). Recall that for architectures with separate input and output channels for each well (**Fig. 22**), $m + n^2$ valves or pumps are required in this situation. Again, the valve-less manifold (240) can be replaced by a valved manifold [e.g., (260) of **Fig. 28**] to better control the diffusion of fluids between the m sources. Flow through well (j,k) is effected when input valve j and output valve k are opened. Alternatively, the same functionality can be achieved with m reservoirs at atmospheric pressure (220) and a negative pressure waste reservoir (either 320 or 340) as in **Fig. 28** by means of $1 \times n$ input (400) and $n \times 1$ output valve (410) manifolds (Fig. 30).

channel k or set of column channels(s) $\{k^*\}$ that allow passing of a segment of liquid or vapor from a given row j to a waste reservoir without going through the wells $\{(j,k)\}$

or the column channels {k}. These additional column channels {k*} are referred to as rinse channels (130), because they enable the rinsing of the row channels without requiring flow through the wells.

In general, a rinse channel k* (130) can be located adjacent and parallel to column channel k (016) but be linked by a via to row channel j (014) at the point were they cross [and not be linked directly to the corresponding well (j,k)]. In one embodiment, every column channel k has an associated parallel rinse channel k* (**Figs. 33** and **34**). In other embodiments, there is only one rinse channel to provide rinsing for the entire set of row channels {j}, or there is any combination of rinse channels {k*} interspersed within the array of column channels {k}. When there is only one rinse channel, it may be preferred to have it located adjacent to the last column channel of the array, where k = n.

Figure 35 shows, in a case of fluid flow through well (j,k), an example of a method to rinse a segment of liquid or vapor from row channel j and into rinse channel k*, where k* is immediately adjacent to column channel k. This procedure and architecture allows this fluid segment to be directed to the waste reservoir without passing over wells {(j, k+1), (j, k+2), etc.} that are downstream along row channel j. This is desirable to limit the diffusion of a given compound from one well (j,k) to other wells downstream {(j, k+1), (j, k+2), etc.} where the compound may not be required. Additionally, as described below, diffusion may be controlled by introducing vapor segments into the microchannel microwell array.

Figure 32 shows how the pumping and valving scheme of Fig. 29, employing positive pressure source reservoirs, can be extended to work with a crossed array of n row channels and 2n column channels (n ordinary column channels plus n rinse column channels). This requires a 2n x 1 output valve manifold (420). In general, for every additional rinse channel k* that is added, one additional valve is added to the valve manifold connected to the column channels. Similarly, the scheme of Fig. 30, employing a negative pressure waste reservoir, can be extended to work with an array incorporating rinse channels (130).

Dual-pumped designs

[...] pumping [...] ed by a continuous pressure gradient that exists along the length of the pathway or tubing. Control of this pressure gradient by means of pressures

applied at the inputs and outputs of the fluid pathways requires that there not be leakage of flow across the walls of the pathway. Leakage also compromises the proper dosing of compounds to the wells for drug screening. The sealing of the walls of the pathways must be adequate for the gauge pressures that are likely to be applied. For fluid flow rates of roughly

5   a few millimeters per minute, path lengths not greater than a few centimeters, and channel cross sections on the order of 100 mm x 100 mm, the Hagen-Poiseuille law predicts that the required end-to-end pressure differential ΔP is less than 1 atmosphere (1 atm. $\approx$ 14 pounds per square inch). Nevertheless, considering that various materials and structures known in the art can be implemented for the device of the invention, the possibility of leakage between

10   neighboring wells is minimized by reducing the gauge pressure exerted across the walls of the system. Towards this end, the present device can use positive pressure source reservoirs with a negative pressure waste reservoir (i.e., a "double-pumped" system) to essentially cut in half the maximum gauge pressures which must be applied to achieve a given total end-to-end pressure differential.

15   For a "single-pumped" system, i.e., a system that is open to atmospheric pressure at one end and that is pumped at the other end (either to a positive or to a negative gauge pressure, $\pm\Delta P$), the pressure difference exerted across the walls of the system is a maximum at the location of the pumped reservoir, i.e., (200) of **Figs. 27** and **29**; or (320, 340) of **Figs. 28 and 30.** Leakage is most likely to occur at these points of maximum gauge pressure, with

20   positive pressure tending to expel fluid out of the pathway and negative pressure tending to draw fluid into the pathway from a neighboring well or channel. (This assumes that the neighboring channels and wells are held at zero gauge pressure when not subjected to flow. Another method to control the pressure differentials across the system walls is to apply matching pressures to the neighboring pathways while they are not under flow.) At

25   intermediate points along the fluid pathway, the pressure differential across the system walls has values between $\pm\Delta P$ and zero for positive and negatively pumped systems, respectively.

The same end-to-end pressure differential ΔP is exerted across the pathway by establishing pressures $\pm\Delta P/2$ at the input and output reservoirs, respectively In this case, the

.... gauge pressure within the device is close to zero if the wells are roughly at the midpoint between the input and output reservoirs along the length of the pathway. At the endpoints of the pathway, the maximum

pressure differences $\pm\Delta P/2$ are more readily held without leakage within plastic tubing or silica capillaries. Detection of leakage is facilitated because it is likely to occur only at the system endpoints.

In one embodiment, the two syringes of a dual pumping system are the same diameter and volume, and because they are linked to the same actuator in a push-pull configuration, each syringe is moved the same linear distance, and identical volumes are exchanged for each increment of movement. As any particular source compound is pushed into the array, an equal volume of waste fluid is pulled out. Thus, the array of *m* source syringes also contains an array of *m* negative pressure waste reservoirs. This array of negative pressure waste reservoirs is multiplexed to the column channels by a *1 x m* valve-less manifold, just as the array of source reservoirs is multiplexed to the row columns.

In another embodiment, the source and waste syringes are of different diameter, and the syringes must be controlled by two separate drivers that drive each syringe at a different linear rate, but yield the same volume changes in each syringe. For example, a smaller diameter syringe could be used for a compound, while a larger diameter syringe could be used as the waste receptacle. If the diameter ratio is a factor of 2, as the driver of the small syringe moves one linear unit to expel fluid, the driver of the large syringe moves only 1/4 linear unit to withdraw fluid, but the volumes exchanged are identical. **Figure 31** shows one particular case of this embodiment of a "dual-pumped" system, in which there is only one waste syringe pump that must be actuated in coordinated fashion (e.g., by means of computer control) with the actuation of any particular source syringe pump. Alternatively, the negative pressure pump may be a capillary action pump, also shown in **Fig. 31**. For the capillary action pump, the "actuation" of the pump is achieved by means of valves.

In summary, the dual-pumped system has the following advantages:

1) reduction in the maximum applied gauge pressure,

2) the applied gauge pressure is small near the midpoint of the pathway, and

3) leakage is readily detected and most likely to occur at the system endpoints.

. . . multi pool flow manifolds between the pumped reservoirs and the crossed channel array allows *m* different pumps (200) or reservoirs (220) in these various

embodiments, with *m* different liquid or vapor mixtures to selectively and sequentially produce flow in each well *(j,k)* of the *n x n* well array (002). More generally, a set of wells defining a single fluid pathway {(j,k), (j,n), (m,k), (m,n) … etc.} can be defined by this system. Flow of only one liquid or vapor mixture can occur through only one fluid pathway

5   at a time. Higher level multiplexing, allowing simultaneous flow of different liquid or vapor mixtures through different pathways, is possible with a more complex system of flow manifolds. Such a system is easily constructed as a generalization of the present design.

Control of diffusion

10   The principle of this embodiment is that the valve element (520) and the valve seat (540) are shaped to allow flow in one direction and to suppress diffusion when flow is stopped.

       Furthermore, the restoring forces on all valves are applied "passively" and constantly. No detailed control system for the check valves is needed, because the control of fluidic

15   pressure gradients is the means by which individual valves are opened.

       The use of crossed microchannels with a large number of open pathways between, for example, the **2n** microchannels and the n^2 wells, may in some embodiments of the invention be augmented by additional methods to control the diffusion of compounds between wells and microchannels. Unlike prior art systems, the microfluidic system of the present

20   invention does not need a detailed control system within the device for control of well-to-well diffusion. Two exemplary means of controlling inter-well diffusion are disclosed that do not require the active control of on-board valves. Both of these methods block inter-well diffusion by means of fluid flow control from valves, pumps, and manifolds that are external to the array of wells, and thus conform to the overall design principles of the invention: the

25   optimal imaging of sub-arrays of wells in a class of fluidic architectures that requires fewer channels and permits smaller inter-well spacings.

       The first method uses the insertion of segments of air between different fluid segments. The positive and/or negative pressure methods given in the above embodiments

can separate air segments in the input channels {j} between a number of different fluids. In particular, appropriate sequencing can direct one fluidic segment to one well (j,k)

and direct another fluidic segment to another well (j, k-1), while also inserting and maintaining an air segment in row j between these two wells. Subsequently, the air segment can be passed out of the system through an unused well or via a rinse channel k* (130)(**Fig. 35**).

The second method of diffusion control uses "normally-closed" check valves (500) between every pair of adjacent vias (via k and via k + 1) (180) that connect the channels j to the wells (j, k) and (j, k + 1) (**Fig. 36**). Similarly, check valves may be used in architectures incorporating rinse column channels {k*}. In other embodiments, check valves may be placed within the vias (160, 180) that connect wells to microchannels or row channels to rinse column channels. These valves are closed unless a pressure gradient is applied across a fluid pathway of the array. Then only those valves distributed along the selected fluid pathway are pushed open. When flow is stopped and the valves return to the closed position, the valves suppress diffusion of compounds between wells and microchannels, or row channels and rinse channels. The restoring force that closes each valve element (520) in its corresponding valve seat (540) may be provided by any number of methods known in the art. The strength of the restoring force must allow the valves to open upon the application of pressure gradients that yield appropriate flow rates (typically less than 1 cm/min.). Methods for application of restoring forces include mechanical force by the deformation of metallic, semiconductor, or organic materials; pneumatic pressure; electrostatic force, magnetic force, and the force of gravity.

In another embodiment, an externally-applied magnetic field gradient (620) induces a restoring force on valve elements (520) that incorporate a magnetic material. This includes but is not limited to the use of paramagnetic beads (600) (e.g., supplied by Dynal Corp.) as shown in **Fig. 37**. These paramagnetic beads (600) serve as balls in micro ball valves that are fabricated within and along each row and column channel. U.S. Patent Nos. 5,643,738 and 5,681,484 describe a magnetically-actuated microball check valve. However, these patents particularly describe an actively-controlled valve that would permit closure of the ball valve "on demand," and has as its purpose to stop flow that is imposed by some external pressure. In the instant case, the magnetic valves would be used for a check valve function.

The movement of selected balls (whenever it is desired to open the valves) along a fluid pathway is achieved by means of causing the fluid

itself to flow. The ball valve is controlled by the flow of fluid, not the flow of fluid is controlled by the ball valve. Moreover, in this embodiment, the valve seat is particularly designed so that a seal is not obtained in one direction, as opposed to the other direction, where a mild seal sufficient to stop diffusion is obtained.

5   In one embodiment, one micro ball valve is located between every pair of adjacent vias (via k and via k + 1) that connect the channels j to the wells (j, k) and (j, k + 1). Each micro ball valve opens only when a pressure gradient is applied across a pathway that includes that valve. The design of the valve seat (540) on one side is shaped (e.g., with grooves) so that flow is not stopped when fluidic pressure moves the ball in the direction of

10   flow. When no pressure gradient is applied (and no fluid is flowing) at the site of a particular ball valve, a magnetic restoring force presses that paramagnetic ball back against the other side of the valve seat (540)(where the seat is shaped to fit the ball) to suppress diffusion of compounds across that portion of the microchannel. This restoring force is induced by a magnetic field gradient applied to the entire array, so that all valves are normally closed.

15   In another embodiment, an externally-applied magnetic field gradient (620) induces a restoring force on valve elements (520) that incorporate a ferromagnetic material. For example precision chrome steel balls are available from Glenn Mills Inc., Clifton, NJ in a range of sub-mm to few mm diameters.

           Fig. 38 shows an embodiment of a shaped valve seat that exemplifies this design

20   principle. Specifically, diffusion past the ball is stopped when the externally-applied restoring force presses the ball to the left into a round opening in the valve seat. When flow is actuated by externally-applied pressures, the ball is forced to the right, but the ball does not stop the flow because the right side of the valve seat is oval shaped and includes a groove-like deformation at along one side, forming path for fluid to bypass the ball in this open

25   position of the valve.

           Fig. 39 shows an embodiment that allows a single magnetic field gradient applied to the whole cassette of the invention to induce force on both a set of ball valves of the input channels and a set of ball valves of the output channels

           experiences a component of force in the direction of the "closed" side of its corresponding valve seat (540). In other embodiments, the magnetic field gradient is applied perpendicular

to the plane of the array, and the ball valves operate in the vertical direction within the horizontal microchannels or within the vertical vias. In the latter case (vertically-actuated ball valves within vertical vias) the via architecture must again allow each ball to be subjected to a component of magnetic force in the direction of the "closed" side of its

5   corresponding valve seat (540).

An example has been given of a ferromagnetic or paramagnetic valve element (520) of spherical shape, but other shapes and other restoring forces may generally be used that function in the same manner.

10 **Well dimensions and characteristics**

In another aspect, the present invention provides a cassette for cell screening, comprising a substrate having a surface, wherein the surface contains a plurality of cell binding locations; a fluid delivery system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that

15 mates with the substrate, wherein the multi-level chamber comprises a crossed array of microfluidic input channels and output channels and a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels; and a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location, and wherein the wells are present at a density of at least

20 about 20 wells per square centimeter. In a preferred embodiment, the well density is between about 20 wells per square centimeter and about 6400 wells per square centimeter.

In a preferred embodiment, the cassette of this aspect of the invention further comprises one or more input manifolds in fluid connection with the microfluidic input channels one or more output manifolds in fluid connection with the microfluidic output

25 channels. In another preferred embodiment, the cassette of this aspect of the invention further comprises at least one source receptacle in fluid connection with the one or more input manifolds and at least one waste receptacle in fluid connection with one or more output manifolds. The various control devices in these embodiments are as described above.

No limitation is placed on the ...

... minimum distance and area between wells in the array, this shape has the disadvantage of potential differences in cell culture or fluidic properties in the corner regions

of the wells. Therefore, a well shape that is circular or that is rectangular with rounded corners is used in a preferred embodiment of the invention.

The array system of the present invention is designed for the imaging of individual cells within a field of cells in a drug screening assay system. Screening with imaging assays that involve the measurement of each individual cell within a field of cells, in parallel, is referred to as High Content Screening (HCS), because detailed information about intracellular processes is contained within the image of a single cell. Screening with lower resolution imaging or with detectors that integrate a signal over a population of cells is referred to as High Throughput Screening (HTS), because it is faster. In HCS, the cells are typically identified by imaging the nuclei of the cells, which are spherical or ellipsoidal in shape with a long-axis length that ranges typically from 5 μm to 15 μm. The cell array used in the present invention optimizes the rate of HCS by using a higher-density multi-well format compared to conventional 96-well plates.

For HCS, the number of cells that provide a statistically-relevant sample as well as the number of cells that are required per unit surface area varies depending on the type of assay and the type of cell being cultured. Thus, the minimum size of the well required by statistical criteria for each assay is different. Nevertheless, no matter what that minimum or optimum well size is, the rate at which wells can be read with adequate image resolution to resolve individual cell nuclei is optimized by the present device, which allows several wells to be imaged at one time in parallel. This is done most efficiently if there is minimal wasted space between wells, and the cell-plated area within the entire image is therefore maximized.

Moreover, the present array system optimizes the flexible implementation of both HTS and HCS on the same optical imaging cell-based screening system. The required pixel density (pixels per micron) is lower for HTS (for example, 0.001 to 0.1 pixels per micron) and higher for HCS (for example, 0.1 to 4.0 or more pixels per micron). The present system provides seamless, combined use of HTS and HCS, where a "hit" identified in HTS is also read in HCS mode for more detailed analysis. In fact, the availability of HCS on the same platform and with the same sample, greatly increases the information associated with "hits" identified in the HTS mode.

... required for HCS in combination with the maximum well density of the array are the two critical parameters that define the maximum rate at which wells can be read in the HCS

mode. This invention describes how the design of the present array system can maximize this HCS rate, as well as the HTS rate.

In use with 96-well microplates, a conventional microscope imaging system can image square fields that are 0.1 mm to 10 mm wide centered on each 7-mm diameter well. The wells are imaged or "read" in series. At each position of the stage, typically, only one area of a well is read. To read the entire array (either in HTS or HCS modes) the sample stage must move the microplateat least 96 times.

In this invention, we define a novel cell screening method in which many wells are read at one time. Because the present array has well widths and inter-well distances that are roughly 10 times smaller than in the 96-well microplate (wells that are sub-millimeter in width and overall plate dimensions of several millimeters rather than several centimeters wide), a cell screening system, such as that disclosed in the present invention, can image many wells simultaneously. Thus, the sample stage is moved fewer times per plate and several wells are read in parallel (Fig. 39).

This essentially involves the serial imaging of "sub-arrays" (020) of the entire microwell array (040), while acquiring data from all wells of each sub-array in parallel. For example, the imaging of 3 x 3 well sub-arrays requires 9 times fewer movements of the sample stage, and allows a roughly 9-times faster rate of data acquisition.

For a given CCD detector array (080), the magnification of the optical system (060) determines the area of the sub-array (020) that is projected onto the detector. For example, an image pixel density of 1 pixel per micron is achieved for a 1000 μm x 1000 μm sub-array of wells by using 10x optical magnification and a 1000 x 1000 pixel CCD array of 10 μm x 10 μm elements. In a second example, a 2000 μm x 2000 μm sub-array of wells can be imaged onto a 2000 x 2000 pixel CCD array at the same magnification to yield the same image pixel density. In a third example, an image pixel density of 0.1 pixel per micron is achieved for a 10mm x 10mm sub-array of wells by using 1.0x optical magnification and a 1000 x 1000 pixel CCD array of 10 μm x 10 μm elements. In a fourth example, a 10mm x 10mm sub-array of wells can be imaged onto a 2000 x 2000 pixel CCD array at 2.0x

... the present invention that optimize the imaging of sub-arrays. These well sizes must be large enough to contain a desired number of cells bound to a desired number of cell

binding sites within each location of the array. Due to the considerable range of desired cell densities to be cultured on the cell binding sites (i.e.: cultures of very widely spaced cells, or of confluent monolayers may be desired), the desired well size may range from that which contains only one cell on a very small cell binding site of about 10 μm in diameter (i.e.: a

5   well size of 20 to 50 microns), to that which contains either one large cell binding site or an array of many cell binding sites (i.e.: a well size of 1 to 2 mm) comprising a single location of the array. The former case leads to higher well densities, and the latter to lower well densities.

       For both high and low well densities, and for wells that contain one or many cell

10   binding sites, the class of microfluidic architectures according to the present invention permit the optimal spacing of wells, the minimal wasted space between the wells, and the maximum number of wells that can be simultaneously imaged in a sub-array and still obtain adequate pixel resolution in the image.

       We now use the four examples of optical resolution given above to illustrate how for

15   a preferred range of sub-array sizes, optical magnifications, and pixel resolutions, and based on the advantages of the microfluidic architecture described herein, the present device supports well sizes and spacings that enable great increases in the speed at which the wells can be imaged or read. Additional sub-array sizes, optical magnifications, and pixel resolutions are supported by the methods and devices of the invention. The scope of the

20   invention is not limited by the current state of the art in the number of available pixels, nor in the pixel sizes in electronic imaging systems.

1. For the first example above, a 1000 μm x 1000 μm sub-array of wells is imaged onto a 1000 x 1000 pixel CCD camera (with image resolution of 1 pixel per micron). We now give four examples of well sizes and densities that are supported by this invention. (a)

25   First, a 2 x 2 sub-array of 300 μm x 300 μm wells with 200 μm-thick walls and a well density of 400 wells per cm^2. Imaging these wells in groups of 4 yields a 4-fold speed increase compared to reading the plate one well at a time. (b) Second, a 3 x 3 subarray of 200 μm x 200 μm wells with 100 μm walls of this

       current highest density commercial microplate (the 1536 well plate). (c) Third, a 5 x 5 sub-array of 100 μm x 100 μm wells and 100 μm walls yields a 25-fold speed increase and a density of 2500 wells per cm^2 (d) Fourth, for an even higher

density of wells and a greater speed advantage, the well can be 25 μm x 25 μm with 100 μm walls, yielding an 8 x 8 sub-array, a 64-fold speed increase, and a well density of 6400 cells per cm².

2.   For a 2000 μm x 2000 μm sub-array of wells imaged onto a 2000 x 2000 pixel CCD camera (with image resolution of 1 pixel per micron), one additional example of well density is considered in addition to the four examples considered above. (a) First, a 2 x 2 sub-array of 800 μm x 800 μm wells with 200 μm-thick walls yields a 4-fold speed increase and a well density of 100 wells per cm². The number of wells per unit area is a factor of 5 greater than that provided by the current highest density commercial microplate (the 1536 well plate). (b) Second, a 4 x 4 sub-array of 300 μm x 300 μm wells with 200 μm-thick walls yields a 16-fold speed increase and a well density of 400 wells per cm². (c) Third, a 6 x 6 sub-array of 200 μm x 200 μm wells with 100 μm walls yields a 36-fold speed increase and a well density of 900 wells per cm². (d) Fourth, a 10 x 10 sub-array of 100 μm x 100 μm wells with 100 μm walls yields a 100-fold speed increase and a well density of 2500 wells per cm². (e) Fifth, a 16 x 16 sub-array of 25 μM x 25 μM wells with 100 μm walls yields a 256-fold speed increase and a well density of 6400 wells per cm².

3.   For a 10 mm x 10 mm sub-array of wells imaged onto a 1000 x 1000 pixel CCD camera (with image resolution of 0.1 pixel per micron), we again describe the four cases of well sizes and densities described in example 1 above. (a) First, a 20 x 20 sub-array of 300 μm x 300 μm wells with 200 μm-thick walls yields a 400-fold speed increase and a density of 400 wells per cm². (b) Second, a 30 x 30 sub-array of 200 μm x 200 μm wells with 100 μm walls yields a 900-fold speed increase and a well density of 1111 wells per cm². As before, this case supports a well density 80 times greater than that provided by the current highest density commercial microplate. (c) Third, a 50 x 50 sub-array of 100 μm x 100 μm wells with 100 μm walls yields a 2500-fold speed increase and a well density of 2500 wells per cm². (d) Fourth, an 80 x 80 sub-array of 25 μm x 25 μm wells with 100 μm walls separating the wells that are 100 μm wide, yielding 2500 wells per sub-array, and 2500 wells per cm².

4. For a 10 mm x 10 mm sub-array of wells imaged onto a 2000 x 2000 pixel CCD camera (with image resolution of 0.1 pixel per micron), the cases from example 2 above of 100, 400, 1111, 2500, and 6400 wells cm^2 again apply, but yield 100 times greater speed increases because the subarrays are 10 times wider on each side. Thus, the speed increases for well densities of 100, 400, 1111, 2500, and 6400 wells/cm2 are 400X, 1600X, 3600X, 10,000X, and 25,600X, respectively, in the 10 mm x 10 mm array.

The following table compares well pitches for various other devices:

Type of plate	Reference	Well to well distance (mm)	Wells/cm^2	Estimated Speed increase*
Standard 1536 well plate		2.25	20	
12 x 12 array in 1.5" x 1.5" area	Gen. Engr. News **18:12** (1998)	3	11	
864 well plate: 24 x 26 array in std. microplate footprint of 108 cm^2	US 5,910,287	3	11	
9600 well plate: 80 x 120 array in a std. microplate footprint of 108 cm^2	US 5,910,287	0.9	123	
	Present invention			
1 mm x 1mm subarray: 1000 x 1000 pixel CCD	Example 1(a)	0.5	400	4X
	Example 1(b)	0.3	1111	9X
	Example 1(c)	0.2	2500	25X
	Example 1(d)	0.125	6400	64X
	Example 2(b)	0.5	400	16X
	Example 2(c)	0.3	1111	36X
	Example 2(d)	0.2	2500	100X

	Example 2(e)	0.125	6400	256X
10 mm x 10 mm subarray: 1000 x 1000 pixel CCD	Example 3(a)	0.5	400	400X
	Example 3(b)	0.3	1111	900X
	Example 3(c)	0.2	2500	2500X
	Example 3(d)	0.125	6400	6400X
10 mm x 10 mm subarray: 2000 x 2000 pixel CCD	Example 4(a)	1	100	400X
	Example 4(b)	0.5	400	1600X
	Example 4(c)	0.3	1111	3600X
	Example 4(d)	0.2	2500	10,000X
	Example 4(e)	0.125	6400	25,600X

*Estimated speed increase is a comparison of the speed at which the entire array could be imaged by sub-arrays compared to imaging by separately imaging each well

5   All of these particular aspects of the instant microfluidic device – the crossed-channel, multilevel architectures, the high well density, the fluid flow control into these architectures, the arrangement of wells into spatially-optimized sub-arrays of wells, and the optional means of control of inter-well diffusion of compounds – are specifically selected and designed to form an integrated system that is compatible with the use of cell culture medium (a polar, 10   aqueous solution containing biological macromolecules and salts), with the maintenance of desired, physiological levels of dissolved oxygen and carbon dioxide gases in the medium, and with the maintenance of desired, physiological temperature (typically 37° C, but other temperatures in the approximate range of 15° C to 40° C may be desired for particular cell types).

15   Particul...

... ... when using cell ... tissues and ... after the medium is moved through the microchannels and to the wells. The sealing of these microchannels and wells from the atmosphere enables the partial

pressures of gasses to be controlled by means of the equilibration of the external vessels. In a preferred embodiment, the level of carbon dioxide in the media within the wells may be established and maintained by equilibrating the media prior to its flow into the device, and/or by allowing exchange of a mixture of carbon dioxide and air with the media as it sits within the well.   A number of different environmentally controlled chambers for live cell culture exist. (U.S. Patent Nos. 5,552,321 and 4,974,952; Payne et al., J. Microscopy 147:329-335 (1987); Boltz et al., Cytometry 17:128-134 (1994); Moores et al., Proc. Natl. Acad. Sci. 93:443-446 (1996); Nature Biotech. 14:3621-362 (1996)).

In a further preferred embodiment, the temperature of the entire substrate is controlled by being in contact with, or composed integrally with, a heater and with a temperature sensor. An electronic temperature control system regulates the heater to attain a chosen temperature set point. Thus, the design of the microfluidic array system described in this invention is readily used in a way that supports live cell culture without an external incubator system.   **Figure 12** shows how this invention provides for the automated reading of cassettes. The cassettes are kept under controlled environmental conditions within two storage compartments (**48** and **54**) before and after reading in the luminescence reader instrument **44**.   While being read by the luminescence reader instrument, the system maintains the appropriate temperature and composition of dissolved gasses in the cell culture media within the wells of the cassette. Temperature control within the wells is provided by heating device(s) and temperature sensor(s) within the cassette of the luminescence reader instrument.   Control of dissolved gas composition within the wells is provided by equilibration of the media with a source of pre-mixed air and carbon dioxide (available commercially) prior to the flow of media into the wells of the cassette. Any type of controller for regulating gas partial pressures may be used with the instant invention. In a preferred embodiment, the system comprises a gas controller comprising a pre-mixed gas source connected to a reservoir or reservoirs containing cell culture media and/or test compounds. A gas pressure regulator and flow control valve control the rate of flow of this gas mixture and its pressure in the connected fluid reservoir(s). This equilibration of the gas mixture with the fluids may be carried out in reservoir or reservoirs.

present invention provides the need in the art for devices and methods that decrease the amount of time necessary to conduct high throughput and/or high content cell-

based screening. The devices of the invention are also ideally suited as a cell support system for a hand-held diagnostic device (i.e.: a miniaturized imaging cell-based assay system). The drug discovery industry already uses 96-well microplates and is in transition towards the use of 384-well plates. Plates with up to 1536 wells are envisioned. Thus, there is a great

5 advantage in both throughput and economy from the use of even higher density plates, such as that of the present invention.

The sealed containment of the cells in the cell array of the instant invention will provide a rugged system that is portable and usable in any orientation. For military and civilian toxin testing, the devices of the instant invention will provide two primary

10 advantages. First, toxin testing based on cellular function is advantageous compared to toxin testing based on molecular structure (e.g., mass spectrometry or optical spectroscopies), because it tests the ultimate effect of the compound on tissue rather than a related property of the compound whose link to toxicity may be unknown or dependent on other conditions. Second, an automated, miniaturized, sturdy, and portable format for cellular-function based

15 sensor would be advantageous compared to the current state-of-the-art that involves large microplates filled by large robotic pipetting systems and read by large microscopic readers.

Additionally, other assay capabilities can be integrated into the device of the present invention that are not possible in prior art devices, such as simple plastic microplates. For example, a mass spectrometric or capillary electrophoretic analytical device, or systems for

20 DNA and/or protein analysis, can be integrated in the instant device to measure chemical and structural parameters of test compounds. Such integration would provide information that is complementary to the cell-based, functional parameters measured by a HCS or HTS system. For conventional microplates, such additional assay functions are external to the plate, and therefore require extensive additional equipment for both the transfer of samples from the

25 microplate and for the analytical equipment itself. In the miniaturized format with integrated fluidics of the present invention, the sample is integrally pumped to a microanalytical system on the chip or on an integrally-connected second chip. Such microanalytical chips will reduce the cost and dramatically increase the speed of cell-based analysis. In this context, the marriage of a mini

Example 4.   Cell Patterning

Polymeric and glass surfaces in their native structures have been used as cellular growth substrates for decades. Differing techniques have been utilized to adjust the surface chemistry of these materials to make them more attractive for cell adhesion, including adsorption of cell adhesion molecules; sulfonisation of the material (European Patent Application 576184); co-polymer blends of extracellular matrix protein fragments such as RGD (U.S. Patent No. 5,733,538); and chemical oxidation (using solution chemistry) of the surface for further chemical modification (using solution chemistry) (U.S. Patent No. 5,330,911) such as silanes (U.S. Patent No. 5,077,085) or thiols (U.S. Patent No. 5,776,748).

In addition to adjusting the surface of these substrates to render them more attractive for cellular adhesion, techniques have been developed to render the surfaces repulsive for cellular adhesion. Cell repulsive surfaces have been achieved by utilizing extremely hydrophobic surfaces (contact angle >90°), which show cell repulsion for several days. (Dulcey et al., Science 252:551 (1991)). A more widely utilized method employs extremely hydrophilic surfaces (contact angle approximately 0°), by immobilizing sugars and oxygen-rich moieties to the surface through common linking chemistries. These surfaces demonstrate longer periods of cell repulsion before degrading and allowing cell adhesion. The most utilized molecule for cell repulsion is oxygen-rich poly(ethylene glycol) (PEG). PEG can be attached to polymeric and glass substrates by methods including chemically activating the substrate to react with a poly(ethylene imide)-PEG molecule (Brink, Colloids and Surfaces, 1992. 66: p. 149-156), aminating an activated surface and reacting it with bifunctional electrophilic molecules such as PEG-epoxide (Bergstrom, K., Journal of Biomedical Materials Research, 1992. 26: p. 779-790; WO 98/32466 EP0576184, Nilsson, D., in Methods in Enzymology, 1984. 104: p. 56-69; Sofia, S., Macromolecules, 1998. 31: p. 5059-5070), and photochemically through photolabile PEG-conjugates such as PEG-benzophenone and PEG-styrene co-polymer blends. (Becker, Makromolecular Chemistry, 1982. 3: p. 217-223; U.S. Patent No. 5,502,107)

The techniques mentioned so far will lead to homo-monolayers, containing one of the cell attractive

and cell repulsive

be controlled to a high degree, cells can be patterned on the substrate of choice. Cell patterning has been achieved on glass and metalized glass substrates utilizing silanes (U.S.

Patent No. 5,077,085), thiols (U.S. Patent No. 5,776,748), and azidos (U.S. Patent No. 5,593,814). These methods provide selective localization of cells using a multi-step, equipment intensive process, and/or irreproducible techniques such as deep ultraviolet ablation of molecules, and/or printing by mechanical stamping, and/or require a polymer

5   layer tens of nanometers (nm) in thickness, which changes the optical quality of the substrate.

Thus, there is a need in the art for affordable, facile, equipment insensitive, reproducible methods for cell patterning on durable substrates such as glass and plastic that do not decrease the optical quality of the substrate, as well as for the cell patterning substrates themselves. Most surface modification processes for glass and silicon wafers are not

10   amenable to plastic due to the nature of the harsh solvents used. Thiols are unsuitable for coating on plastics as they require a coinage metal for forming a coordination bond with the substrate. Silanes, though amenable to coating on plastics, require a hydroxylated surface, such as presented by glass and silicon, to form a covalent bond with the substrate.

Thus, in one aspect, the present invention provides novel methods for making a

15   substrate for selective cell patterning. In one embodiment, the method comprises

a)   providing a substrate with a surface, wherein the surface contains reactive hydroxyl groups;

b)   contacting the hydroxyl groups on the surface of the substrate with a bifunctional molecule comprising a hydroxy-reactive moiety and a nucleophilic moiety to

20   form a monolayer;

c)   applying a stencil to the substrate;

d)   applying an electrophilic cell repulsive moiety to exposed regions of the monolayer to form a covalent bond between the cell repulsive moiety and the bifunctional nucleophile deposited in step b to form cell repulsive locations;

25   e)   removing the stencil; and

f)   applying cell adhesive molecules to the substrate to produce cell binding locations, wherein the cell adhesion molecules bind the substrate only in positions that were contacted by the stencil.

30

surface, wherein the surface comprises reactive hydroxyl groups on the surface of the substrate;

b)   contacting the hydroxyl groups on the surface of the substrate with a bifunctional molecule comprising a hydroxy-reactive moiety and a nucleophilic moiety to form a monolayer

c)   applying a stencil to the substrate;

d)   applying cell adhesive molecules to exposed regions on the monolayer to produce cell binding locations;

e)   removing the stencil; and

f)   applying an electrophilic cell repulsive moiety to the substrate to produce cell repulsive locations, wherein the cell repulsive moiety forms a covalent bond with the bifunctional nucleophile deposited in step b only in positions that were contacted by the stencil.

The surface reactive hydroxyl groups can either be naturally occurring, or can be introduced via any technique known in the art. For example, polymer or glass can be oxidized so that they present surface hydroxyl groups, which react with organosilanes to produce covalent Si-O-substrate (siloxane) linkages. (U.S. Patent No. 5,077,085)

In a preferred embodiment, oxidation is accomplished by oxygen plasma treatment, which can be achieved using oxygen doped radio frequency glow discharge. This discharge is accomplished with an instrument that can produce charged particles (electrons and positive ions) that interact with the background gas (oxygen), to produce free radicals under the time-varying electric field in radio frequency. The sample is placed into a cylindrical reactor, a minimal amount of oxygen gas is introduced, and charged particles are evolved between parallel-plated electrodes resulting in the cleavage of the O_2 bond. After this cleavage, high-energy free radicals can insert themselves into the polymer backbone resulting in the formation of various oxygen moieties, among which are hydroxyl groups. (U.S. Patent No. 5,357,005; U.S. Patent No. 5,132,108)

As used herein, the bifunctional molecule comprises

(a)   a hydroxyl-reactive electrophile, including but not limited to silanes, carboxymethyl groups

(b)   a nucleophile, including but not limited to sulfhydryl groups, amine groups, hydroxyl groups, or proteins or fragments thereof, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells.

In one embodiment, the bifunctional molecule comprises an organosilane, wherein silane is the electrophile, and the nucleophile includes, but is not limited to sulfhydryl groups, amine groups, hydroxyl groups, or proteins, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells. As used herein, organosilanes fall into a larger class of molecules, which have the capability of forming self-assembled (SA) films. The general form of this molecule comprises R_nSiX_{4-n}, where $n = 1,2$, or 3; $X = Cl$, OCH_3, or OC_2H_5, and R is the nucleophile as described above.

In a preferred embodiment, the bifunctional molecule comprises an aminosilane, wherein silane is the electrophile that attaches to the surface hydroxyl groups, and an amine group is the nucleophile that can bind to other molecules and/or cells.

In a more preferred embodiment, the aminosilane is selected from the group consisting of methoxy or ethoxy silanes, which include but are not limited to trimethoxysilylpropyldiethylenetriamine,   trimethoxysilylethylenediamine, aminopropyltriethoxysilane, trimethoxyaminopropylsilane, or chlorosilanes such as trichlorosilylethylenediamine, aminopropyltrichlorosilane. In a most preferred embodiment, the amino silane is trimethoxysilylpropyldiethylenetriamine

As used herein, a cell adhesive molecule includes compounds that: (1) introduce charge; and/or (2) are polar; and/or (3) contain sulfur and/or amines; and/or (4) are capable of tethering cells or other cell binding moieties, such as proteins, peptides, and synthetic ligands for cell surface receptors, thereby creating a cell binding location.

As used herein, the term cell repulsive moiety includes compounds that are capable of directly inhibiting cell binding, or that bind to other moieties which inhibit cell binding to the location, including polyethylene glycol (PEG) and other oxygen-rich compounds, sugars, hydrogels, extremely hydrophilic surfaces, or extremely hydrophobic surfaces.

. In all of these embodiments, the cell adhesive molecule and/or cell repulsive moiety

example, vapor phase deposition of various silanes has been demonstrated. (Tripp et al., Langmuir 8:1120-1126 (1992); Moses et al., Analytical Chemistry 20:4 (1978)

In most cases, rather than adding the sample to a solution of silane, a hydroxylated surface is placed in the presence of vaporized silane (achievable by traditional vacuum techniques). The reaction takes place at the surface and results in self-assembled monolayers similar to that of silane solution deposition.

5   A wider range of cell adhesive molecules and cell repulsive moieties can be used with vapor phase deposition, because a solvent is not needed. For example, many silane solvents would dissolve the polymeric substrate and destroy its optical quality. In this embodiment, the method circumvents the use of solvents altogether.

           In another preferred embodiment, the cell repulsive moiety comprises an amine-

10   reactive moiety, including but not limited to 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride)-activated polyethylene glycol (PEG), polyvinylpyrrolidone, polyvinylalcohol, or any other amine-reactive extremely hydrophilic compound such as sugars (mannitol) or PEG, where the amine-reactive part can include, but is not limited to, carboxymethyl groups, succinimides, succinimidyl succinates, benzotriazole carbonates, glycidyl ethers (or

15   epoxides), oxycarbonylimidazoles, p-nitrophenylcarbonates, aldehydes, isocyanates, and tresylates; or any amine-reactive extremely hydrophobic compound such as tridecafluoro-1,1,2,2-tetrahydrooctyl groups (13f) where the amine-reactive part can include, but is not limited to carboxymethyl groups, succinimides, succinimydil succinates, benzotriazole carbonates, glycidyl ethers (or epoxides), oxycarbonylimidazoles, p-nitrophenylcarbonates,

20   aldehydes, isocyanates, and tresylates. In a most preferred embodiment, the amine-reactive cell repulsive moiety comprises tresyl chloride-activated polyethylene glycol ("tresyl-chloride activated-PEG").

           The chemistry of the tresyl-activated PEG can be used to regulate surface hydroxyl, amine, or sulfhydryl groups. Tresyl chloride will allow stable linkages to be formed between

25   the nucleophile and the initial hydroxyl, amine, or sulfhydryl group carrying carbon. In a preferred embodiment, PEG is attached to a tresyl group for reaction with surface aminosilane groups.

           In these preferred embodiments, cell adhesive cues can be defined by the use of a stencil which h...

           ...ed with ... adhesive protein, pr... fragments, or peptides, and seeded with cells resulting in a patterned array of cells.

This hydroxylated substrate is contacted with a bifunctional molecule comprising an electrophile and a nucleophile. This modified substrate is contacted with a textured elastomeric substrate (herein referred to as a 'stencil'), such as rubber, polyurethanes and poly(dimethyl) siloxanes ("PDMS"), to form a hermetic seal between defined regions of the stencil and the modified substrate. In a preferred embodiment, the stencil comprises PDMS. These materials are quite affordable, providing a significant benefit over traditional UV photolithography methods that employ a costly, high energy laser apparatus.   (U.S. Patent No. 5,077,085)

The stencil comprises a physical mask that enables physical protection of defined regions of the underlying substrate from the subsequent solution or vapor phase deposition of the cell repulsive or cell adhesive moiety. This disclosed method of using a 'physical mask' distinguishes itself from existing art that relies on the use of an 'optical mask' (Dulcey et al., Science 252:551 (1991) and U.S. Patent Nos. 5,965,305 and 5,391,463) or 'contact imprinting' (U.S. Patent Nos. 5,512,131 and 5,776,748). The use of optical masks for protecting or de-protecting defined regions of a substrate is limited to the use of photoactivatible chemistries and/or photolabile molecules. The use of 'contact imprinting' is limited to solution phase transfer of materials onto a surface while not enabling 'protection' or 'de-protection' of defined regions of the surface. Further, contact imprinting does not enable reproducible transfer of controlled amounts of material onto the surface. The use of a 'stencil', as disclosed in this invention, allows for protection of a region of the substrate to enable modification of unprotected regions with solution or vapor phase chemistries not limited to photoreactive/photolabile molecules.

The present invention is not constrained to one particular kind of substrate. The tethering chemistry of the primary monolayer, or the organosilane, is such that it reacts with surface hydroxyl groups. These hydroxyl groups can be introduced on the surface of virtually any plastic and glass by low temperature plasma treatment. The secondary tethering chemistry, tresyl chemistry, can react with surface amines, hydroxyl, and sulfhydryls, making it possible to attach to a wider array of surface chemistry. The desired effect is also

these benefits make the disclosed method of patterning on glass and plastics affordable, facile, and accurate.

The benign nature of the chemistry employed makes it attractive for biological applications, allowing the array to be prepared on glass and any thermoplastic and thermoset of choice including, but not limited to poly(styrene), poly(olefin), poly(dimethyl) siloxane (PDMS), poly(carbonate), poly(vinyl) chloride, poly(ethylene), poly(ethylene) terapthalate, Teflon, and fluoronated ethylene co-poly(propylene) (FEP). The present methods also have the ease and flexibility to be applied to polymeric and glass substrates using the same method. Plastics such as poly(styrene), acrylics, and poly(olefin) have benefits over glass, ceramics and metals because of their affordability, flexibility of shape and size, ease of engineering, durability, low cost and control over its optical quality. The plastics are easily obtained at a minimal cost, can be molded into almost any shape conceivable, and are durable.

The present methods for preparing a substrate for selective cell patterning are more reproducible than are methods that employ contact printing, because there is less opportunity for operator error. There is operator dependence when contact printing due to the subjectivity of applying the stamp to the substrate (force by which the stamp is depressed, amount of solution on the stamp) and so the results will vary. (U.S. Patent No. 5,776,748) The present method of using a stencil for masking while performing solution or vapor phase deposition of the cell adhesive molecules and/or cell repulsive moieties is operator independent, thus providing a scalable and manufacturable process.

The instantly disclosed method of cell patterning has a marked advantage over prior thiol chemistry. Previous technology of contact printing with thiols not only introduces operator error, but also requires a thin layer of gold to be evaporated on the surface of the tissue culture substrate. Due to the high temperature involved with gold evaporation, most plastics cannot be used. Optical quality is constrained and fluorescence light is absorbed due to the added layer of gold, which reduces the quality of information gathered when conducting cell-based screening. In addition to a lower optical quality, there is a high cost associated with gold coating. Furthermore, silane linkages are covalent, and are not subject to degradation, as are thiols on gold, which degrade over time due to impurities and the fact that a thiol bond is coordinate . . . .

. . . .

. . . substrate, . . . that one has . . . freedom . . . choose the most superior affordable plastic or glass for optical quality.   In a particular embodiment of the

present method, oxygen plasma is used to activate the surface in the case of poly(styrene) and poly(olefin), and acid washing is used to activate the surface in the case of glass. Both surfaces can be further incubated with a mildly acidic alcoholic solution of aminosilane featuring a primary amine on the terminating end of the tethered molecule. Following silane

5   treatment, a stencil is applied to the substrate. An aqueous solution of tresyl chloride-activated PEG is applied to the substrate around the stencil resulting in regions of exposed amine, and regions of PEG in carefully controlled proximity to one another. After surface modification, the surface can be primed with cell adhesive proteins, protein fragments, or peptides to speed the cell adhesion process. (U.S. Patent No. 5,874,219)

10   In another aspect, the present invention provides novel patterned substrates for cell culture. In one aspect, the invention provides cell patterning substrates, comprising:

     1.   at least a first portion having a reactive surface to which a plurality of cell adhesive molecules are coupled;
     2.   and at least a second portion having an exposed surface to which a plurality of
15   cell repulsive moieties are coupled; wherein the cell adhesive molecules are selected from the group consisting of silanes, and wherein the cell repulsive moieties comprise tresyl-chloride activated poly(ethylene) glycol.

     In a preferred embodiment, the silane comprises R_nSiX_{4-n}, where n = 1,2, or 3; X = Cl,
20   OCH_3, or OC_2H_5; R = a nucleophile, including but not limited to sulfhydryl groups, amine groups, hydroxyl groups, charged groups, polar groups, or proteins, protein fragments, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells. In a preferred embodiment, the silane is an aminosilane. In a more preferred embodiment, the aminosilane is selected from the group consisting of
25   methoxy   or   ethoxy   silanes,   which   include   but   are   not   limited   to trimethoxysilylpropyldiethylenetriamine,   trimethoxysilylethylenediamine, aminopropyltriethoxysilane,   trimethoxyaminopropyl-silane,   or   chlorosilanes   such   as trichlorosilylethylenediamine, aminopropyltrichlorosilane. In a most preferred embodiment, trimethoxysilylpropyldiethylenetriamine is used.

30   In another embodiment, the substrate further comprises cell adhesive proteins, protein fragments, or peptides, wherein the reactivity of the cell adhesion promoters can be modified by varying the composition of the cell adhesive proteins, protein fragments, or peptides. In a further embodiment, the substrate

further comprises cells bound to the cell binding locations, either directly or indirectly via cell adhesive proteins, protein fragments, or peptides. Any cell type may be used, including prokaryotic, eukaryotic, and archaebacterial cells.

5   The cell binding locations according to the various methods and substrates of the invention can be as small as the diameter of a single cell and as large as several hundred cell diameters. The distance between cell binding locations (i.e.: the'cell repulsive locations) is cell size dependent, but is sufficiently large so that a cell cannot bridge the gap between cell binding locations (i.e.: 1 cell diameter), unless a particular application calls for interaction of cells in different cell binding locations.

10   In a further embodiment, the various cell patterning substrates are mated with a fluid delivery system to provide fluid and/or reagent flow to the cell binding location. In a preferred embodiment, the fluid delivery system is that described herein.

In another embodiment, the cell patterning substrate comprises a cell patterning substrate made by the methods of the invention, as disclosed above.

15   This aspect of the present invention may be better understood with reference to the accompanying preferred embodiments that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

20   **Materials and Methods**:

Reagents and instrumentation that can be utilized in carrying out the methods of the invention include, but are not limited to, 60 and 35 mm petri dishes, microplates, thermoplastics, poly(olefin), plasma cleaner/sterilizer, digital convection gauges, trimethoxysilylpropyldiethylenetriamine, and 2,2,2-trifluoroethanesylphonyl-

25   poly(ethylene)$_{5000}$ glycol.

Poly(styrene), poly(olefin), or other thermoplastic substrates such as poly(esters) and poly(ether) are oxygen plasma treated inside a plasma cleaner using the following method. Substrates are placed inside the glass tube chamber and the chamber is evacuated to a pressure of 200 mtorr

...een pulse is repeated 2 more times. After the last oxygen pulse, the gas is allowed to bleed constantly into the chamber, and the final equilibrium pressure (with the oxygen bleed

valve on and the vacuum pump activated) should be ~300 mtorr. After the proper pressure is reached, the voltage switch is turned up to HI (100W) and the substrates are treated for 25 minutes.

5   Glass surfaces are activated using the following method. A 1M KOH solution is prepared in double distilled/deionized (DI) water. The glass surfaces are incubated for 10 minutes in 1M KOH. After 10 minutes, the substrates are rinsed 3 times in double DI water. Coverslips are soaked in HCl:MeOH (1:1) for 30 minutes. After the incubation, the coverslips are rinsed in double DI water, and transferred into a concentrated bath of sulfuric acid for 30 minutes, followed by 3 rinses with double DI water. The coverslips are then

10   boiled in distilled water for 15 minutes, and the surfaces blown dry with a nitrogen gun.

          Aminosilane treatment is the same for glass, poly(styrene), and poly(olefin). A 1% solution of trimethoxysilylpropyldiethylenetriamine is prepared in mildly acidified methanol (94% methanol, 5% water, and 0.004% glacial acetic acid) and incubated with the substrates for 15 minutes. Following silane treatment, the substrates are rinsed with methanol and

15   baked in a 80°C oven for 30 minutes.

          The PDMS stencil is applied to the aminated glass or poly(styrene) (this embodiment includes but is not limited to 50, 100, 200 micron and 500 micron spots). Pressure is applied until the stencil makes a tight seal.

          Tresyl-PEG treatment is the same for glass and poly(styrene). After stencil application,

20   a 0.12M sodium bicarbonate solution is prepared in water. A 25% solution of tresyl-PEG (by weight) is prepared in the bicarbonate. The solution is applied around the stencil and allowed to pool around the PDMS, resulting in the liquid touching only exposed aminated surface areas. The substrates are incubated for 4 hours.

          Following PEG treatment, the surfaces are rinsed with the 0.12M sodium bicarbonate

25   solution. The substrates are allowed to incubate for 2 hours and rinsed under a stream of PBS. The substrates ("partially active") can be stored for more than 30 days in a dry box before protein coating and cell incubation.

          As used herein, the term "partially active" substrates are glass or plastic substrates chemically modified

Protein coating

  1. The partially active substrates are incubated in a protein, protein fragment, or peptide solution, including but not limited to fibronectin, laminin, collagen, vitronectin, osteopontin, or fragments thereof, as well as RGD peptides, RGDS peptides, YIGSR

5 peptides, at concentrations ranging from 1 µg/ml to 25 µg/ml, for 1-2 hours.

  2. Post-incubation, the substrates are rinsed in a stream of PBS.

  3. The protein/peptide coated substrates are lyophilized to preserve the protein/peptide structure. These "quasi-active" substrates can be stored in a dry box for >30 days. The silane linkages are covalent and not subject to degradation, as are thiols on gold,

10 which degrade over time due to impurities and the fact that a thiol bond is coordinate and not covalent. As used herein, the term "quasi-active" substrates are glass or plastic substrates chemically and/or texturally modified to yield a patterned array of protein or peptide-rich domains separated by cytophobic domains.

15

Cell Seeding

  1. Cells are prepared for seeding by standard techniques.

  2. Partially active or quasi-active substrates are re-hydrated in cell culture medium for 5 minutes.

20 3. The cells are incubated at any desired seeding density with the partially or quasi active substrate for 30 minutes to 2 hours (depending on the cell type) in complete cell culture media at 37°C and 5% CO_2.

  4. The "fully active" substrates, arrayed with the cells, can then be used for cell screening assays. After cryopreservation or room temperature desiccation the fully active

25 substrates can be stored long term. As used herein, the term "fully active" substrates are glass or plastic substrates chemically and/or texturally modified to yield a patterned array of cells separated by cytophobic domains. They can be derived from either partially or quasi-active substrates, although the use of quasi-active substrates is preferred.

Stem cells possess the intrinsic ability to: (1) undergo self renewal, or (2) produce differentiated progeny. Extrinsic factors (culture medium, growth factors, physico-chemical

cues from the surrounding cellular milieu) mediate the developmental fate of stem cells. Tissue specific stem cells, also called determined stem cells, also exhibit pluripotency but not totipotency.   Determined stem cells provide the ability to access a primary, partially committed cell that can be driven to either self-replication in culture, or selectively

5   differentiated into a multitude of tissue specific progeny that in-vitro are close, genotypically and phenotypically, to the whole organism, in contrast to immortalized cells that are both genotypically and phenotypically far removed from their precursor cells in the organism.

The pharmacological relevance of using stem cell derived tissue-specific progeny over immortalized cells results from the genotypic and phenotypic match afforded by the

10   former. As a new chemical compound goes from being identified as "active" in a primary drug screen, it must pass through numerous tests designed to assess its pharmacological profile.   Indices of bio-relevance such as cytotoxicity, bioavailablity, and specificity are evaluated together with potency to generate this profile, and require the use of cells genotypically and phenotypically matched to the organism. This increases the probability

15   that the compound will be therapeutically relevant when it gets to the clinic beyond the animal testing stage.   The use of primary cells (hepatocytes, neurons, chondrocytes, myocytes, adipocytes) are well documented in secondary and tertiary testing. However, the difficulty in obtaining the proper cells (access or amount), and culturing them in sufficient quantity to cover assay capacity remains a major problem. The use of stem cell derived

20   primary cells can afford a solution to both higher relevance and a source of primary cells.

Other work has focused on the production of spatially oriented neo-vascular capillaries from endothelial cells that are bound to cell adhesion promoters patterned on a substrate. (Rudolph et al., U.S. Patent No. 5,721,131) However, the resulting substrate contained only a single type of differentiated cell, because the method did not permit

25   individually addressing the cell binding locations with differentiating reagents.

The present invention provides methods for preparing patterned cell substrates comprising a multitude of terminally differentiated cells from an ordered array of stem cells, as well as the substrates themselves. The use of these patterned cell substrates for drug discovery increases the pace of lead compound investigation.

The methods and substrate of the present invention mimic events in developmental biology: formation of committed mature or terminally differentiated cells from stem cells

using controlled delivery of differentiation factors (including, but not limited to extracellular matrix (ECM) derivatized substrates, autocrine/paracrine/endocrine factors, etc.). Prior technology teaches induction of cellular heterogeneity by "peppering" a cell substrate surface with a multitude of cell adhesive domains enabling selective adhesion of one or more immortalized, genotypically and phenotypically distinct cell types from solution. The limited number of "cell-specific cell adhesive moieties" limits the number of distinct cell types attainable on a single substrate. Prior technology does not take advantage of the intrinsic ability of stem cells to differentiate into a multitude of differentiated progeny.

In contrast, the present invention teaches induction of cellular heterogeneity on any substrate of choice (ceramics, metals, polymers, composites etc.) by the controlled differentiation of population(s) of stem cells into a multitude of differentiated cells. The controlled differentiation enables creation of "patterns of mixed cell types" in any number of variations and geometry. The present invention also enables creation of organ specific and tissue specific cell substrates that are close in genotypic and phenotypic relevance to the organism of choice, as well as substrates that model in vivo interactions between tissue specific and/or organ specific cells. This enables creation of a cell based screening platform capable of providing information with greater relevance to the organism or systemic level.

The preparation cell substrate of the present invention requires providing a patterned stem cell substrate, comprising:

   (a)   a substrate surface;
   (b)   a plurality of cell binding locations on the surface, wherein a cell binding location comprises:
          (1)   one or more cell adhesive molecules; and
          (2)   a plurality of stem cells bound to the cell adhesive molecules; and
   (c)   a plurality of cell repulsive locations on the surface, wherein the cell repulsive locations comprise a cell repulsive moiety , wherein individual cell binding locations are separated by cell repulsive locations.

A single type, or multiple types of stem cells are bound to the cell binding locations in a patterned array. As used herein, the term "patterned stem cell substrate" means that the

positioning of the different stem cell types is also controlled.

As used herein, the term "stem cells" refers to any cell type that possesses the ability to produce at least one type of differentiated progeny. As such, stem cells include, but are not limited to cells capable of differentiation into any cell type and self-renewal (pluripotent cells); cells capable of differentiation into any cell type but not self-renewal (totipotent cells);

5   cells capable of self-renewal and differentiation into many cell types (broad potential; multipotent stem cells), cells capable of limited self-renewal and differentiation into limited types of cells (progenitor cells); and cells that have committed to differentiation into a specific cell type, but have not yet completed differentiation (committed progenitor cells). (See, for example, Gage et al. Science 287:1433-1438 (2000).) In various embodiments, the

10 stem cells are selected from the group consisting of neural stem cells, neural progenitor cells, glial progenitor cells, mesenchymal stem cells, hematopoietic stem cells, epithelial stem cells, hepatic stem cells, embryonic stem cells, or combinations thereof. Reference to specific classes of stem cells is provided below:

Hepatic stem cells are reviewed in Thorgeirsson, *FASEB J.* 10:1249 (1996).

15 Examples of hepatic stem cells include, but are not limited to, SEC cells, oval cells, as well as cultured WB-F344 cells, L2039 cells, and RLEΦ13 cells (Coleman et al., *Am. J. Pathol.* 151:353 (1997); Omori et al., *Hepatology* 26:720 (1997); Fiorino et al., In Vitro Cell Dev. Biol. Anim. 34:247-258 (1998); Agelli et al., Histochem. J. 29:205-217 (1997); Brill et al., Proc. Soc. Exp. Biol. Med. 204:261-269 (1993)).

20   **Neural stem and progenitor cells:** Examples of neuronal stem and progenitor cells include, but are not limited to, NT-2 cells (Pleasure et al., J. Neuroscience 12:1802-1815 (1992)), and those described in Gage, Science 287:1433-1438 (2000); Gritti et al., *J. of Neuroscience* 16:1091-1100 (1996); Frederiksen et al., *Neuron* 1:439 (1988); Reynolds and Weiss, *Science* 255:1707 (1992); Davis and Temple, *Nature* 372:263 (1994); McKay, *Science*

25 276:66-71 (1997);. Vicario-Abejon et al., *Neuron* 15:105 (1995); Johe et al., *Genes and Develop.* 10:3129-3142 (1996); and U.S. Patent Nos. 5,824,489; 6,001,654; 6,033,906).

Glial progenitor cells: Examples of glial progenitor cells include, but are not limited to SNB-19 cells and oligodendrocytes that can be diferentiated in vitro (Raible et al., J.

Mesenchymal stem cells are pluripotent progenitor cells that possess the ability to differentiate into a variety of mesenchymal tissue, including bone, cartilage, tendon, muscle,

marrow stroma, fat and dermis. Such stem cells include, but are not limited to, C2C12 cells (Teboul et al., J. Biol. Chem. 270:28183-28187 (1995); Nishimura et al., J. Biol. Chem. 273:1872-1879 (1998)); and cells such as those described in U.S. Patent Nos. 5,486,359; 5,827,740; 5,942,225; 6,022,540.

5   **Hematopoietic stem cells** refer to any hematopoietic pluripotent progenitor cells capable of giving rise to a variety of differentiated hematopoietic cell types. Cell types within this definition include, but are not limited to CD34$^+$ bone marrow mononuclear cells (BMMC) (Berardi, et al., *Blood* 86:2123-2129, 1995), PBSC (Fritsch, et al., *Bone Marrow Transplantation* 17:169-178, 1996), cobblestone area forming cells (CAFC) (Lemieux, et al.,

10   *Blood* 86:1339-1347, 1995) and 5-FU BM cells (Alcorn and Holyoake, *Blood Reviews* 10:167-176, 1996); U.S. Patent No. 5,807,744)

       Epithelial stem cells refer to cells that are long-lived, relatively undifferentiated, have a great potential for cell division, and are ultimately responsible for the homeostasis of epithelium.   Cells of this type include, but are not limited to, those described in U.S. Patent

15   No. 5,556,783; U.S. Patent No. 5,423,778; Rochat et al., *Cell* 76:1063 (1994); Jones et al. *Cell* 73:713 (1993); Jones et al., *Cell* 80:83 (1995)) and Slack, Science 287:1431-1433 (2000).

       The cell adhesive molecules of this aspect of the invention can comprise any compound that is capable of supporting stem cell adhesion to the substrate, such as those

20   described above, including cell adhesion molecules, co-polymer blends of extracellular matrix proteins or protein fragments such as RGD-containing peptides, silanes or thiols. In one embodiment, aminosilanes, such as methoxy or ethoxy silanes as disclosed above , are used.

       The cell repulsive moieties comprise moieties that are capable of directly inhibiting

25   cell binding, or that bind to other moieties which inhibit cell binding to the cell repulsive location, including but not limited to polyethylene glycol (PEG) and other oxygen-rich compounds, sugars, hydrogels, extremely hydrophilic surfaces, or extremely hydrophobic surfaces, as described above.

cell cell differentiation.   Such compounds include, but are not limited to self-assembled monolayers of thiols or silanes coupled to cell adhesive ligands, which are utilized to enable

the creation of cell binding locations, while preventing their uncontrolled differentiation. Another example is the addition of protease thrombin to cultures of the Neuro2A neuroblastoma cell line, which inhibits differentiation of the cells into neurite-containing neuronal cells. (Gurwitz et al., Proc. Natl. Acad. Sci. 85:3440-3444 (1988)) Thus, the cell binding promoter can also serve as an inhibitor of uncontrolled differentiation. Alternatively, the cell binding locations are covered with a feeder layer of cells to inhibit uncontrolled stem cell differentiation.

The substrate of this aspect of the invention can be made of any material known in the art, including but not limited to plastics, glass, ceramics and metals. Preferably, such patterned substrates are made on commercially viable plastic substrates such as polystyrene or poly(olefin). In a preferred embodiment, the substrates possess 100 μm-500 μm diameter circular cell binding locations separated by edge-to-edge spacing ranging from 25 μm to 125 μm. The overall footprint on the substrate will match the format of 96, 384, and 1536 well microplates, and be approximately 150-fold smaller in surface area compared to 1536 (32 rows x 48 columns), 384 (16 rows x 24 columns), and 96 wells (8 rows x 12 columns) microplates.

In a further aspect, the present invention provides a patterned differentiated cell substrate comprising:

(a)   a substrate surface;
(b)   a first plurality of cell binding locations on the surface comprising:
    (i)   one or more cell adhesive molecule;
    (ii)   a first differentiated cell type;
(c)   a second plurality of cell binding locations on the surface comprising:
    (i)   one or more cell adhesive molecule;
    (ii)   a second differentiated cell type; wherein the first and second differentiated cell types are arrayed on the substrate in a controlled pattern; and
(d)   a plurality of cell repulsive locations on the surface, wherein the cell repulsive locations comprise a cell repulsive moiety , wherein individual cell binding locations are separated by cell repulsive locations.

As used herein, the term "patterned differentiated cell substrate" means that the differentiated cells are arrayed in the .....

.... embodiment, the differentiated cells are derived from stem cells that were selectively differentiated on the substrate. In this embodiment, the stem cell type is selected from the group consisting of neural stem cells, neural progenitor cells, glial progenitor cells

mesenchymal stem cells, hematopoietic stem cells, epithelial stem cells, hepatic stem cells, embryonic stem cells, or combinations thereof. Alternatively, differentiation can be initiated prior to plating the cells (committed progenitor cells) on the substrate . One of skill in the art will be able to envision many permutations of this embodiment, including but not limited to the following:

1.   Selective differentiation of a single stem cell type into two or more differentiated cell types in predetermined locations on a single substrate.

2.   Selective differentiation of two or more stem cell types arrayed on a single substrate to generate two or more differentiated cell types.

3.   Sequential, selective differentiation of a single or multiple stem cell types, whereby a single differentiated cell type is produced first, followed by selective differentiation of the stem cell type(s) in other cell binding locations.

Thus, in a preferred embodiment, the present invention results in a substrate with multiple types of differentiated cell types arranged in a pre-determined manner. The number of different cell types that can be arrayed is limited only by the differentiation potential of the stem cell, since the various cell binding locations can be individually addressed with differentiating agents, using devices including, but not limited to microspotters (Cartesian Technologies^{TM}, Hewlett Packard^{TM}, Genetic MicroSystems^{TM}), and fluid delivery system such as, but not limited to those disclosed herein, and in U.S. Patent Nos. 5,858,188; and 6,007,690. Selective addressing of the stem cells with differentiating agents enables controlled differentiation into the progeny of choice. In a preferred embodiment, the fluid delivery system of the present invention is combined with the patterned cell substrate to produce a microfluidic cassette, which can deliver differentiating compounds to the patterned undifferentiated stem cells.

In a preferred embodiment, the first and second differentiated cell types are cell types found in a single tissue, including but not limited to brain, vascular tissue, skin, pancreas, kidney, liver, lung, heart, intestine, and stomach.

In another preferred embodiment, the first

smooth and/or skeletal muscle; epithelial tissue-smooth muscle; vascular endothelium-smooth muscle; glial cells-endothelial cells of blood capillaries; adipose cells-

axons of peripheral nerve cells and/or Schwann cells; and pancreatic B cells-pancreatic A cells.

In another preferred embodiment, the first and second differentiated cell types are selected from the group consisting of glial cells, neurons, adipocytes, smooth muscle cells, skeletal muscle cells, osteoblasts, chondrocytes, stromal cells, and myocytes.

In various most preferred embodiments, (a) the first differentiated cell type derived is a glial cell, and the second differentiated cell type is a neuronal cell, thus producing a cell substrate model for the brain; (b) the first differentiated cell type is an adipocyte and the second differentiated cell type is a muscle cell; (c) the first differentiated cell type a neuronal cell and the second differentiated cell type is a muscle cell.

In a further preferred embodiment, the patterned stem cell substrate or the patterned cell substrate is mated with a fluid delivery system chamber to form a cassette, wherein the fluid delivery system delivers reagents to the stem cells or differentiated cells, and comprises:

(i)   a plurality of domains matching the cell binding locations on the surface of the substrate, and

(ii)   microfluidic channels that supply reagents to the cell binding locations.

As used herein, "reagents" include differentiating agents, as well as cell culture medium and any cell culture supplements for cell culture, and test compounds for screening the effects of drug compound libraries and toxins on the cells.

In a most preferred embodiment, a single microfluidic channel supplies fluid to a single cell binding location on the substrate, to provide separate fluid flow to each cell binding location, thereby permitting selective differentiation of the stem cells, and/or selective treatment of the stem cells or differentiated cells in one or more cell binding locations with a test agent of choice.

In another aspect, the present invention provides a patterned differentiated cell substrate made by a method of selective differentiation of patterned stem cells, wherein the method comprises:

(a)   providing a patterned stem cell substrate

(i)   one or more cell adhesive molecules;

(ii)   a first differentiated cell type;

(3)   a second plurality of cell binding locations on the surface comprising

(i)   one or more cell adhesive molecules ;

(ii)   a second differentiated cell type; wherein the first and second differentiated cell types are arrayed on the substrate in a controlled pattern;

(b)   selectively contacting the cell binding locations with differentiating agents to provide controlled differentiation of the stem cells into a progeny of choice, wherein the selective contacting produces a patterned differentiated cell substrate.

In a preferred embodiment, the patterned cell substrate is mated with a fluid delivery system as described above.

In further preferred embodiments of the above aspects, the stem cells and the resulting differentiated cells possess at least one luminescent reporter molecule, for use in cell screening assays to determine the distribution, environment or activity of the luminescent reporter molecules on or in the cells or within or between subcellular compartments of the cells, in response to a test agent of choice. A variety of such luminescent reporter molecules can be used in this aspect of the invention, including but not limited to those described in U.S. Patent No. 5,989,835; pending U.S. Patent Application Nos. 09/031,271 (filed February 27, 1998); 09/352,171 (filed July 12, 1999); 09/293,209 (filed April 16, 1999); 09/293,209 (filed April 16, 1999); 09/398,965 (filed September 17, 1999); 09/430,656 (filed October 29, 1999); 09/513,783 (filed February 24, 2000); Giuliano et al., Ann. Rev. Biophys. Biomol. Struct. 24:405-434 (1995); and Giuliano et al., Trends in Biotech. 16:135-140 (1998), all references incorporated by reference herein in their entirety.

The luminescent probes can be small molecules, labeled macromolecules, or genetically engineered proteins, including, but not limited to green fluorescent protein chimeras. As used herein, "luminescent" probes include fluorescent, luminescent, and chemiluminescent probes.

In another embodiment, only one of the differentiated cell types possesses a luminescent reporter molecule, and when an interaction occurs between the first and second differentiated cell types, only one of the cell types reports the interaction, via the luminescent reporter molecule.

The luminescently labeled reporter molecule may be expressed by or added to the cells

. . . . luminescently labeled protein chimera in transfected stem cells. Alternatively, the luminescently labeled reporter molecule may be expressed, isolated, and bulk-loaded into the stem cells, or the reporter molecule may be

luminescently labeled after isolation. As a further alternative, the reporter molecule can be expressed by the stem cell, which is subsequently contacted with a luminescent label, such as a labeled antibody, that detects the reporter molecule.

Preferably, the luminescent reporter molecules in the first differentiated cell type are spectrally distinguishable from the luminescent reporter molecules in the second differentiated cell type.

The present invention also provides methods for selective stem cell differentiation, comprising providing a patterned stem cell substrate as disclosed above, and selectively contacting the cell binding locations with differentiating agents to provide controlled differentiation of the stem cells into the progeny of choice, wherein the selective contacting produces a patterned differentiated cell substrate. Any method known in the art for differentiating the stem cells into differentiated cells can be used. References providing conditions for differentiating the various stem cells into differentiated progeny can be found, for example, in the following references, which are incorporated by reference herein in their entirety:

Mesenchymal stem cells: U.S. Patent No. 5,827,740 (adipogenic differentiation); U.S. Patent No. 6,022,540 (osteogenic differentiation); U.S. Patent No. 5,942,225 (osteogenic, chondrogenic, tendenogenic, marrow stromal cell, and myogenic differentiation); Cuenda et al., J. Biol. Chem. 274:4341-4346 (1999) (C2C12 myogenic differentiation); Nishimura et al., J. Biol. Chem. 273:1872-1879 (1998) (C2C12 osteoblastic differentiation); Teboul et al., J. Biol. Chem. 270:28183-28187 (1995) (C2C12 adipogenic differentiation)

Neural stem and progenitor cells: U.S. Patent No. 5,824,489 (neurons and glia); U.S. Patent No. 6,001,654 (neurons and smooth muscle cells); U.S. Patent No. 6,033,906 (glial cells); Pleasure et al., J. Neurosci. 12:1802-1815 (1992) (NTera2 differentiation into neurons);

Glial and neural stem and progenitor cells: Welch et al., In Vitro Cell. Dev. Biol.-Animal 31:610-616 (1995) (SNB-19 glial differentiation); U.S. Patent No. 5,824,489 (neurons and glia)

Hematopoietic

stem cells ...... Zhang et al., Blood 95:138-146 (2000); Zhang et al., Blood 92:118-128 (1998).

Hepatic stem cells: Brill et al., Proc. Soc. Exp. Biol. Med. 204:261-269 (1993); Brill et al., Dig. Dis. Sci., 44:364-371 (1999); Fiorino et al., In Vitro Cell Dev. Biol. Anim. 34:247-258 (1998).

In various preferred embodiments, the substrate is mated with a fluid delivery system as disclosed above, and the stem cells possess at least one luminescent reporter molecule that serves to identify the phenotype of the progeny of choice.

In these embodiments, the stem cells are contacted with differentiating agents, to effect differentiation of the transfected patterned cells into differentiated progeny, using the appropriate differentiating agents applied either homogeneously to the substrate (for single progeny) or selectively to specific cell binding locations (multiple progeny). Differentiation of a single stem cell type into different progeny, or of different stem cells into different progeny, permits the formation of substrates with cell binding locations that bear different cell types in any desired juxtaposition. In this way, simple to moderately complex models of cellular differentiation are used to prepare multicellular tissue-specific and organ-specific cell substrates for use in cell based analysis for drug discovery and life sciences.

In one model system, neuronal and glial stem cells are engineered to express fluorescent protein reporter molecules to measure the dynamics of their cytoskeletal proteins. The cytoskeleton has become a well-characterized and valid drug discovery target for which there are likely to be many lead compounds in the drug discovery pipeline at any one time. Each of the stem cell lines express spectrally distinct reporter molecules such that they can be patterned into separate locations within a cell array as well as be patterned together, (co-cultured) within the same location. Alternatively, the different stem cells may be patterned into separate locations, wherein the locations are in close enough proximity to permit interactions between the cells in the different locations. The latter two aspect allow the simultaneous measurement of drug responses of the two cell types in an organotypic context where the cells are allowed to interact as they would within the brain tissue of a living animal. Because the reporter molecules contained within each cell type are spectrally distinct, the platform detects and assigns function to each cell type within the co-culture.

In a second model system

types, skeletal muscle myocytes and adipocytes. In this case, the stem cells are engineered to express a luminescent reporter molecule of carbohydrate metabolic flux,

including but not limited to reporters of the phosphorylation state of PFK-1 and PFK-2, the measurement of cellular ATP levels (energy charge), the ratio of oxidation-reduction co-factors such as $NAD^{+}/NADH$, and the concentration of the second messenger cAMP, that are measured both in time and in space within each cell type. For muscle and adipose cells,

5   carbohydrate metabolism plays an important role in regulating the physiological function of each cell type; contraction and relaxation of the muscle cells and fat storage and mobilization in adipocytes. Therefore, this model system permits measurement of the effect of lead compounds on the same molecular pathway within two tissue types. Moreover, this multiple tissue type screening platform permits the efficient addressing of lead compound efficacy,

10   specificity, and toxicology.

A third, more complex model system involves the co-cultivation of neuronal and skeletal muscle stem cells with both types being engineered to express spectrally distinct luminescent reporter molecules. The cells are patterned and differentiated on the substrate. The effect of lead compounds on the complex interaction of neurons and skeletal muscle cells

15   is measured using the luminescent reporter molecules engineered into each cell type. The co-cultivation of differentiated neuronal and muscle cells permits direct measurement of excitation-contraction coupling events and the effects that lead compounds have on these events.

These approaches can be generalized to interactions between other tissue types and

20   interactions between multiple cell types within an organ, including but not limited to peripheral nerve-smooth or skeletal muscle; epithelial tissue-smooth muscle; vascular endothelium-smooth muscle; glial cells-endothelial cells of blood capillaries; adipose cells-axons of peripheral nerve cells and Schwann cells; and pancreatic B cells-pancreatic A cells.

The present invention further provides methods for cell based screening, wherein the

25   stem cells and/or differentiated cells possess at least one luminescent reporter molecule, for use in cell screening assays to determine the distribution, environment or activity of the luminescent reporter molecules on or in the cells or within or between subcellular compartments of the cells, in response to a test agent of choice. A variety of such luminescent reporter molecules

(filed February 2, 1998), 09/352,171 (filed July 12, 1999); 09/293,209 (filed April 16, 1999); 09/293,209 (filed April 16, 1999); 09/398,965 (filed September 17,

1999); 09/430,656 (filed October 29, 1999); 09/513,783 (filed February 24, 2000); Giuliano et al., Ann. Rev. Biophys. Biomol. Struct. 24:405-434 (1995); and Giuliano et al., Trends in Biotech. 16:135-140 (1998), all references incorporated by reference herein in their entirety.

In this embodiment of the method, the patterned stem cell arrays and patterned differentiated cell arrays are used to analyze changes in the distribution, environment or activity of the luminescent reporter molecules on or in the cells or within or between subcellular compartments of the cells in response to a test compound. The cells are imaged and/or scanned using a cell screening system comprising an optical system with a stage adapted for holding a substrate containing cells, a detection device that is capable of creating a digital image, a means for directing fluorescence or luminescence emitted from the cells to the detection device, and a computer for receiving and processing data from the detection device. A preferred embodiment of such a device is disclosed in U.S. Patent No. 5,989,835; and pending U.S. Patent Application No. 09/031,271 (filed February 27, 1998), both references incorporated by reference herein in their entirety. Utilizing the cell screening system, luminescent signals from the reporter molecules are converted into digital data; and the digital data is used to determine changes in the distribution, environment or activity of the reporter molecules in response to the test agent.

Such digital data can be used to report the effect of a test compound on distribution of the reporter molecule between: cytoplasm-nucleus, cell membrane-cytoplasm, endoplasmic reticulum-Golgi apparatus, as well as to report on apoptosis; receptor internalization; transcription factor activation; protein kinase activation; protease activity; organelle structure, distribution, and function; macromolecule distribution; gene expression; microtubule cytoskeletal structure; actin cytoskeletal structure; nuclear shape; nuclear area; nuclear size; nuclear perimeter; mitochondrial potential; cell shape; cell motility; cell size; and cell perimeter. (For example, see U.S. Patent No. 5,989,835; pending U.S. Patent Application Nos. 09/031,271 (filed February 27, 1998); 09/352,171 (filed July 12, 1999); 09/293,209 (filed April 16, 1999); 09/398,965 (filed September 17, 1999); 09/430,656 (filed October 29, 1999); and 09/513,783 (filed February 24, 2000).

The use of a flexible laborator...

...teslar... ...chnologies... ... Hewlett Packard ..., Genetic MicroSystems ...), permits the delivery of specific cell binding locations with a differentiating agent of choice.

Examples

Organotypic Differentiation Model Systems

5   *Glial differentiation:* Cells taken from a highly aggressive human glioblastoma tumor have been shown to grow indefinitely in culture and to exhibit altered morphological and growth characteristics in the presence of a differentiation agent. These cells, named SNB-19 (Welch et al., 1995), are engineered to express a luminescent reporter molecule (see below) and patterned onto cell substrates, either by themselves or in combination with neuronal stem

10   cells. To induce differentiation, a mixture of 1 mM dibutyryl-cAMP and 1 mM isobutylmethyl xanthine (a phosphodiesterase inhibitor) is added to the culture, and the cells are allowed to incubate for 12-24 hours. These agents induce the cells to elaborate multiple processes that often interact with other glia in the same culture (Welch et al., 1995), as well as cause the cells to stop dividing.

15   The glial stem cells are transfected to express a green fluorescent protein (GFP)-glial fibrillary acidic protein (GFAP). chimera. GFAP is a component of the intermediate filament cytoskeleton, and is a major cytoskeletal protein found in glial stem cells and differentiated glia.

20   *Neuronal differentiation:* Several neuronal stem cell lines exist that can be used in the instant invention. NT2 cells from a human teratocarcinoma cell line (Pleasure et al., 1992) are unique in that they can be induced to differentiate into stable, post-mitotic human neurons, and they have been shown to be a vehicle for the expression of diverse gene products. (Pleasure et al., 1992) NT2 cells are engineered to express a blue fluorescent

25   protein (BFP)-β-tubulin chimera. β-tubulin is a major component of the cytoskeleton. The cells are then patterned onto cell substrates, either by themselves or in combination with glial stem cells. To induce differentiation, NT2 cells initially enter a program of differentiation that begins with a two week treatment of retinoic acid (10^{-5} M), mitotic inhibitors, and a specialized extracellular matrix. The partially differentiated cells are transferred to the

tional proteins, such as the luminescent protein reporter molecule.

Mixed glial-neuronal differentiation: NT2 stem cells in the final stages of differentiation are added with SNB-19 cells to the same substrate. After both cell types attach, the co-cultures are treated with 1 mM dibutyryl-cAMP and 1 mM isobutylmethyl xanthine. The two cell types are allowed to interact as they differentiate. Because the NT2 cells are committed to differentiation, the dibutyryl-cAMP has little to no effect on neuronal cell differentiation, and may even enhance it, since cAMP is known to induce the differentiation of several cell types.

Adipose and skeletal muscle tissue from a common stem cell: The mouse C2C12 cell line is pluripotent and has been shown capable of differentiating into skeletal muscle (Cuenda and Cohen, 1999), adipocytes (Teboul et al., 1995), and osteoblasts (Nishimura et al., 1998). The C2C12 cells are first engineered to express a luminescent reporter molecule of carbohydrate metabolism (see below). The cells are patterned onto substrates where the growth medium contains <1% calf serum. This large decrease in serum concentration (10% originally) induces the C2C12 cells, over a period of 24-48 hours, to stop dividing, fuse into elongated, multinucleated cells, and form contractile myotubes. To induce adipocyte differentiation, the cells are treated with a mix of 5 μM thiazolidinedione and 100 μM fatty acid (Teboul et al., 1995). Differentiation occurs after 24-48 hours and is accompanied by the slowing of cell growth and the uptake of fatty acids by the cells and their incorporation into lipid droplets.

The C2C12 cells are transfected with a reporter molecule comprising 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2), which plays a key role in balancing cellular energy utilization and storage. The activity of this bifunctional enzyme can act to switch a cell between carbohydrate oxidation (energy yielding) and carbohydrate synthesis (energy requiring). The phosphorylation state of PFK-2 dictates whether the enzyme will stimulate cellular carbohydrate breakdown or synthesis. (Kurland and Pilkis, Protein Science 4:1023-1037 (1995). The amino acid sequence containing the PFK-2 phosphorylation site is inserted into a specific site within the coding sequence for GFP that tolerates insertions (Baird et al., P...

Nerve and muscle tissue interaction: Cellular components from the other model systems are combined to model tissue-tissue interactions. Neuronal NT2 cells are combined with C2C12 cells to allow their interaction during differentiation, much like tissues interact develop during normal development. Three scenarios are tested:

5   1.   NT2 and C2C12 cells are arrayed together and allowed to differentiate together;

       2.   NT2 cells are arrayed on the substrate and differentiated, followed by addition and differentiation of C2C12 cells;

       3.   C2C12 muscle stem cells are arrayed on the substrate and differentiated,
10   followed by addition and differentiation of neuronal NT2 stems.

Other Examples

Neural crest stem cells can be treated with poly-D-lysine and fibronectin to produce
15   peripheral neurons and glia, as disclosed in U.S. Patent No. 6,033,906. The same stem cells when treated with fibronectin only produce glial cells but not neurons.

Mesenchymal stem cells treated with 100 nM dexamethasone + 10 mM β-glycerophosphate are induced to undergo osteogenesis. (U.S. Patent No. 5,942,225) The same stem cells treated with 5 ng/ml BMP are induced to undergo chondrogenesis; if treated
20   with 5-azacytidine, they are induced to undergo myogenesis. Finally, the same stem cells treated with 10 u/ml IL-1α differentiate into stromal cells.

U.S. Patent No. 5,827,740 teaches treating mesenchymal stem cells with glucocorticoids and phosphodiesterase inhibitor to induce adipogenesis.

25   The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.

We claim

5   1.   A method of making a substrate for selective cell patterning comprising:

        a)   providing a substrate with a surface, wherein the surface contains reactive hydroxyl groups;

        b)   contacting the hydroxyl groups on the surface of the substrate with a bifunctional molecule comprising a hydroxy-reactive moiety and a nucleophilic moiety to

10   form a monolayer;

        c)   applying a stencil to the substrate;

        d)   applying an electrophilic cell repulsive moiety to exposed regions of the monolayer to form a covalent bond between the cell repulsive moiety and the bifunctional nucleophile deposited in step b, to produce cell repulsive locations;

15   e)   removing the stencil; and

        f)   applying cell adhesive molecules to the substrate to produce cell binding locations, wherein the cell adhesion molecules bind the substrate only in positions that were contacted by the stencil.

20   2.   A method of making a substrate for selective cell patterning comprising:

        a)   providing a substrate with a surface, wherein the surface comprises reactive hydroxyl groups on the surface of the substrate;

        b)   contacting the hydroxyl groups on the surface of the substrate with a bifunctional molecule comprising a hydroxy-reactive moiety and a nucleophilic moiety to

25   form a monolayer

        c)   applying a stencil to the substrate;

        d)   applying cell adhesive molecules to exposed regions on the monolayer to produce cell binding locations;

        e)   removing the stencil; and

            wherein the cell repulsive moiety forms a covalent bond with the

bifunctional nucleophile deposited in step b only in positions that were contacted by the stencil.

3.   The method of claim 1 or 2 wherein the substrate is treated to provide the reactive hydroxyl groups on the surface of the substrate.

4.   The method of claim 3 wherein the substrate is oxygen plasma treated to provide the reactive hydroxyl groups on the surface of the substrate.

5.   The method of claim 1-2 wherein the bifunctional molecule comprises an organosilane

6.   The method of claim 1-2 wherein the bifunctional molecule comprises an aminosilane.

7 .   The method of claim 1-2 wherein the reactive cell repulsive moiety comprises a tresyl-chloride activated-poly(ethylene) glycol compound.

8 .   The method of claim 1-2 wherein vapor phase deposition is used for the contacting of the hydroxyl groups on the surface of the substrate with a bifunctional molecule.

9 .   The method of claim 1-2 further comprising contacting the cell binding locations with a cell-binding protein or peptide under conditions to permit binding of the cell-binding protein or peptide to the cell binding location.

10 .   The method of claim 1-2 further comprising contacting the cell binding locations with cells under conditions to permit binding of the cells to the cell binding locations.

11   The method of claim 9 or 10

the cell binding protein or peptide

12.   The method of claim 9 further comprising lyophilizing the substrate.

13 .   The method of claim 10 further comprising cryopreserving or desiccating the substrate.

5

14 .   The method of claim 11 further comprising cryopreserving or desiccating the substrate.

15 .   A cell patterning substrate, comprising at least a first portion having a reactive surface to which a first plurality of cell adhesive molecules are coupled, and at least a second portion having an exposed surface to which a second plurality of cell repulsive moieties are coupled, wherein the cell adhesive molecules are selected from the group consisting of silanes, and wherein the cell repulsive moieties comprise tresyl chloride-activated poly(ethylene) glycol.

16 .   The cell patterning substrate of claim 15, wherein the silane comprises an aminosilane.

17 .   The cell patterning substrate of claim 16, wherein the aminosilane is selected from the group consisting of methoxy or ethoxy silanes, which include but are not limited to trimethoxysilylpropyldiethylenetriamine,   trimethoxysilylethylenediamine, aminopropyltriethoxysilane,   trimethoxyaminopropyl-silane,   or   chlorosilanes   such   as trichlorosilylethylenediamine, aminopropyltrichlorosilane.

18.   The cell patterning substrate of claim 16, wherein the aminosilane is trimethoxysilylpropyldiethylenetriamine.

19 .   The cell patterning substrate of claim 15, further comprising cell-binding proteins or peptides bound to the plurality of cell adhesive molecules.

21.   The cell patterning substrate of claim 15, further comprising cells bound to the plurality of cell adhesion promoters.

22.   The cell patterning substrate of claim 19, further comprising cells bound to the cell-binding proteins or peptides.

23.   The cell patterning substrate of claim 21, wherein the substrate is cryopreserved or subjected to desiccation.

24.   The cell patterning substrate of claim 22, wherein the substrate is cryopreserved or subjected to desiccation.

25.   A cell patterning substrate, prepared by the method of claims 1-2.

Figure 1

A

B

Figure 2

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Figure 3A

Rhodamine - Actin

Hoechst -Nuclei
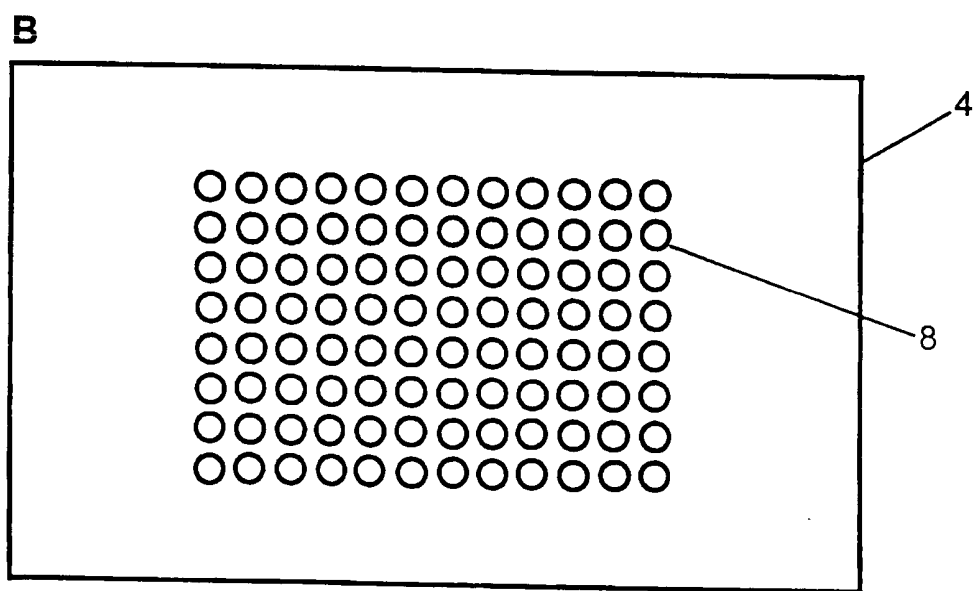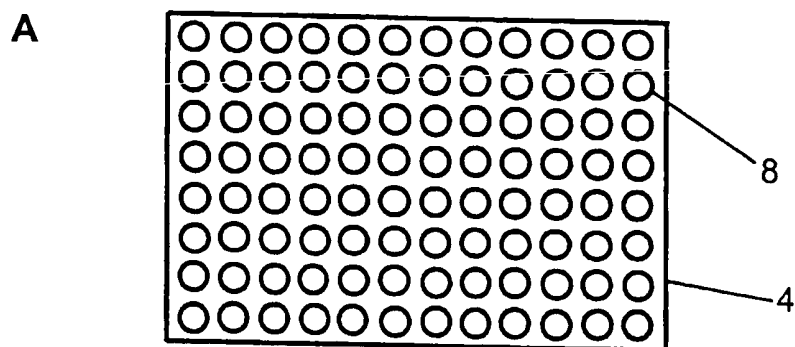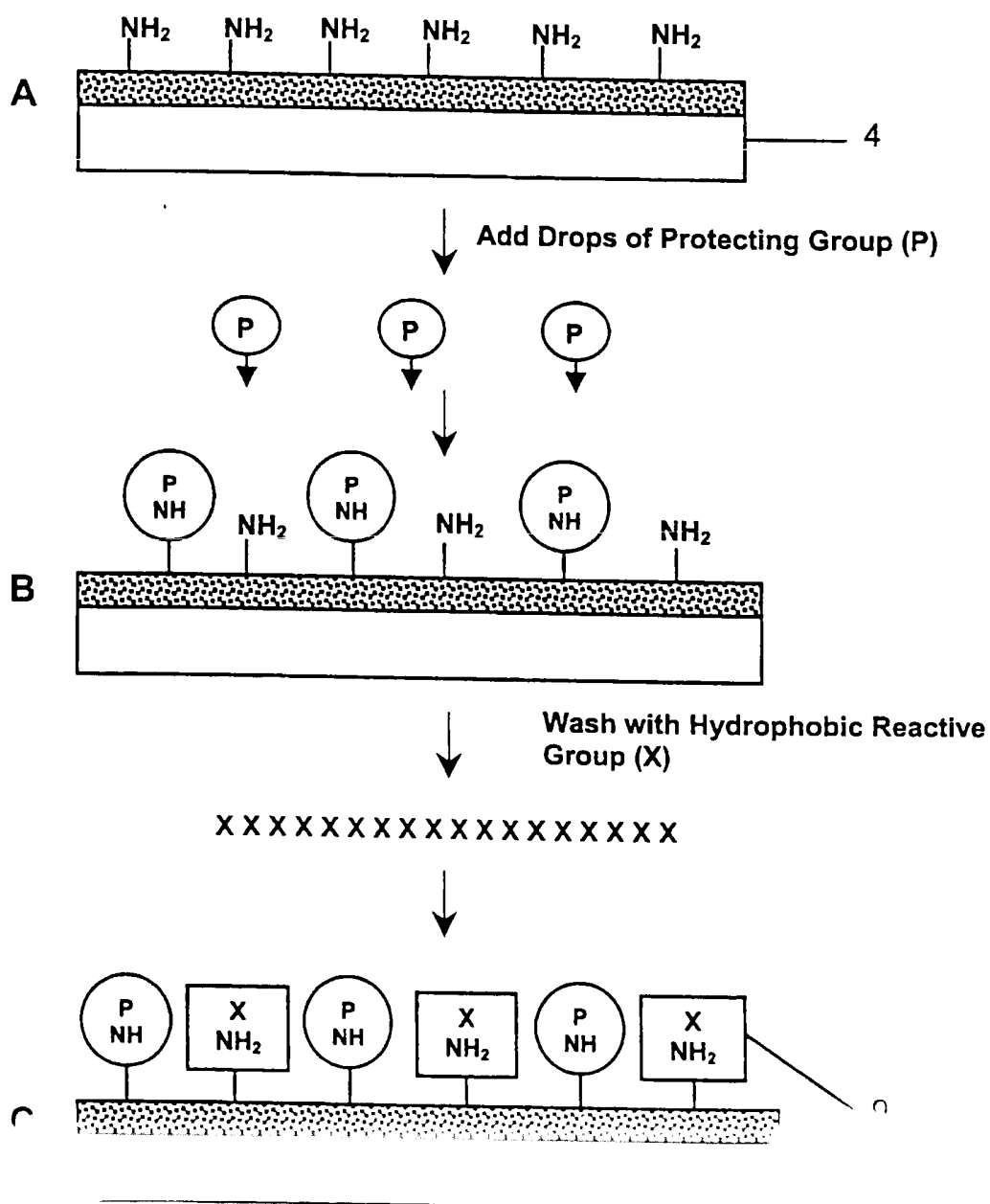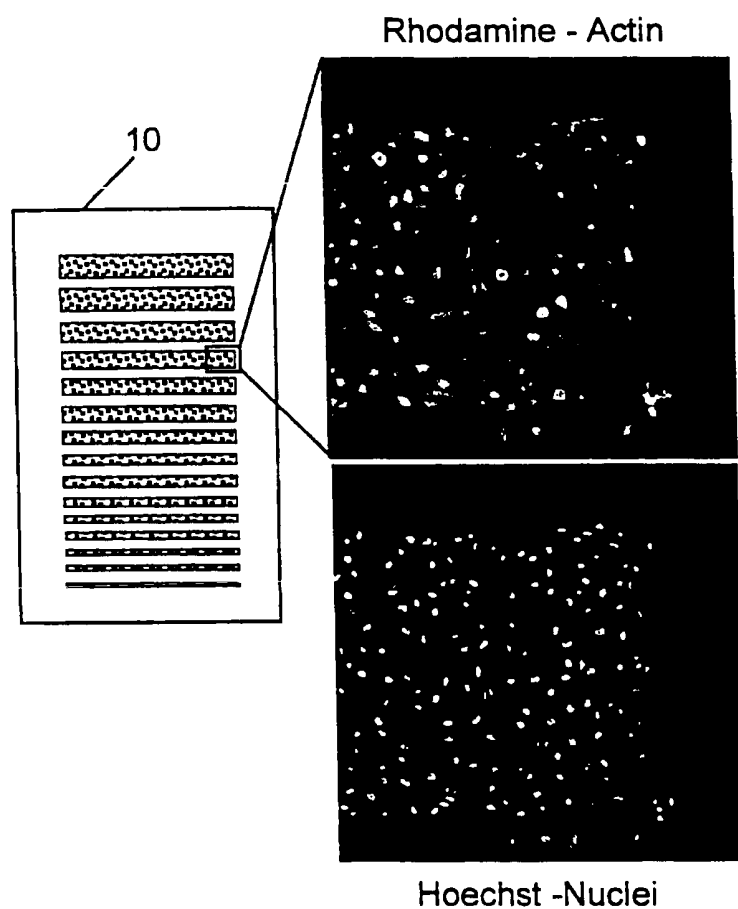
Figure 3B
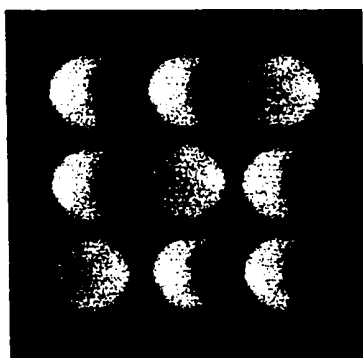
Sodium Fluorescein binding to
Cell Chip to identify the location
of 200 um spot patterns.
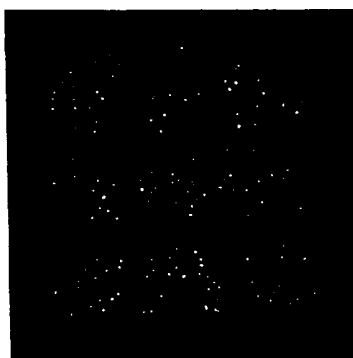
Fluorescent cell attachment
of L929 cells to patterned
spots (low magnification, 4x)

Fluorescent cell attachment
of 3T3 cells to patterned
spots. Nuclei (above), Actin
in same cells (below)
(higher magnification, 20x)
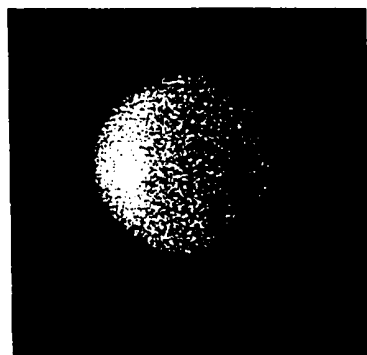
Sodium Fluorescein binding to
Cell chip to identify the location
of 400 um spots patterns.

4/48
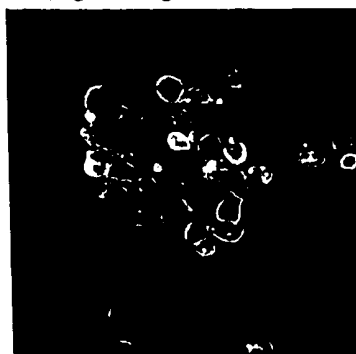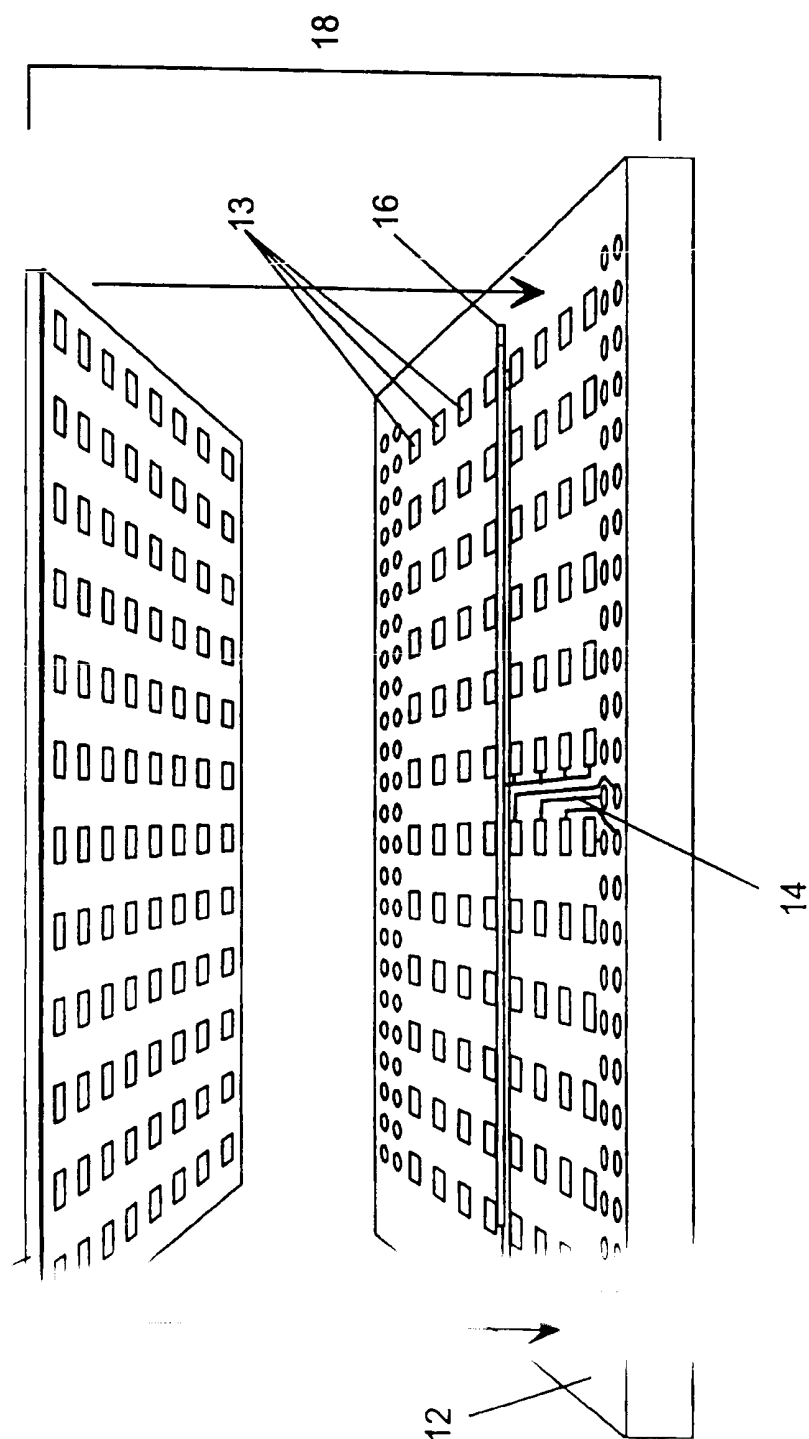
Figure 4

Figure 5

Figure 6

Figure 7

A

B
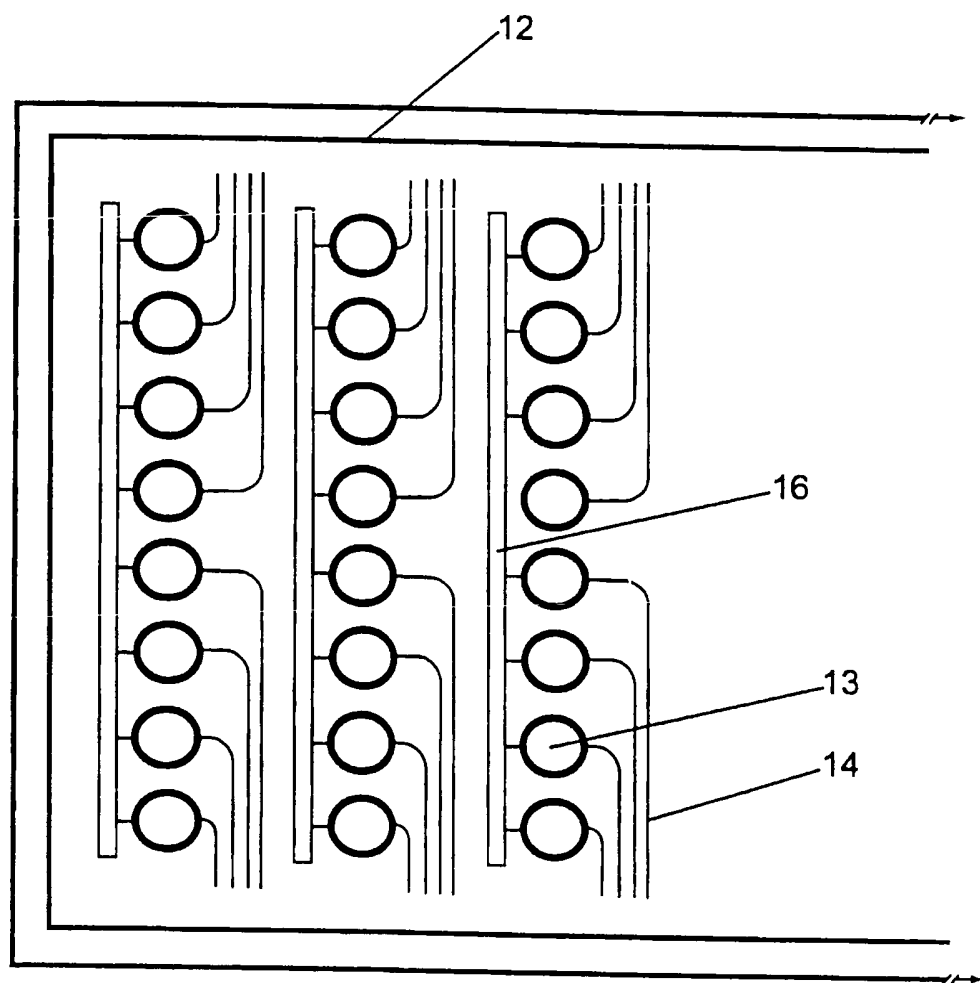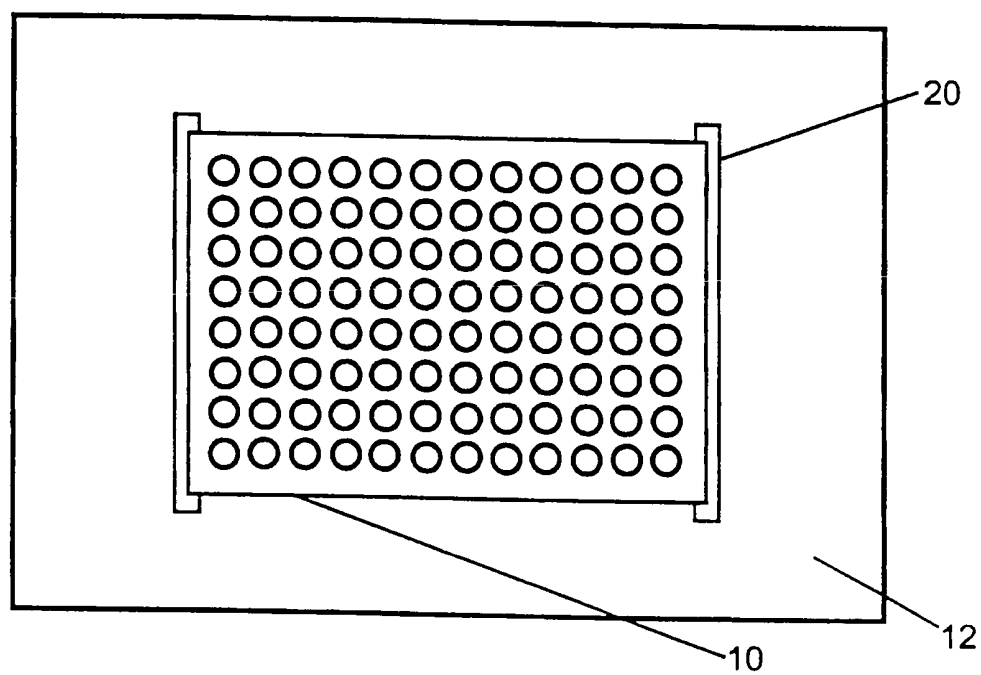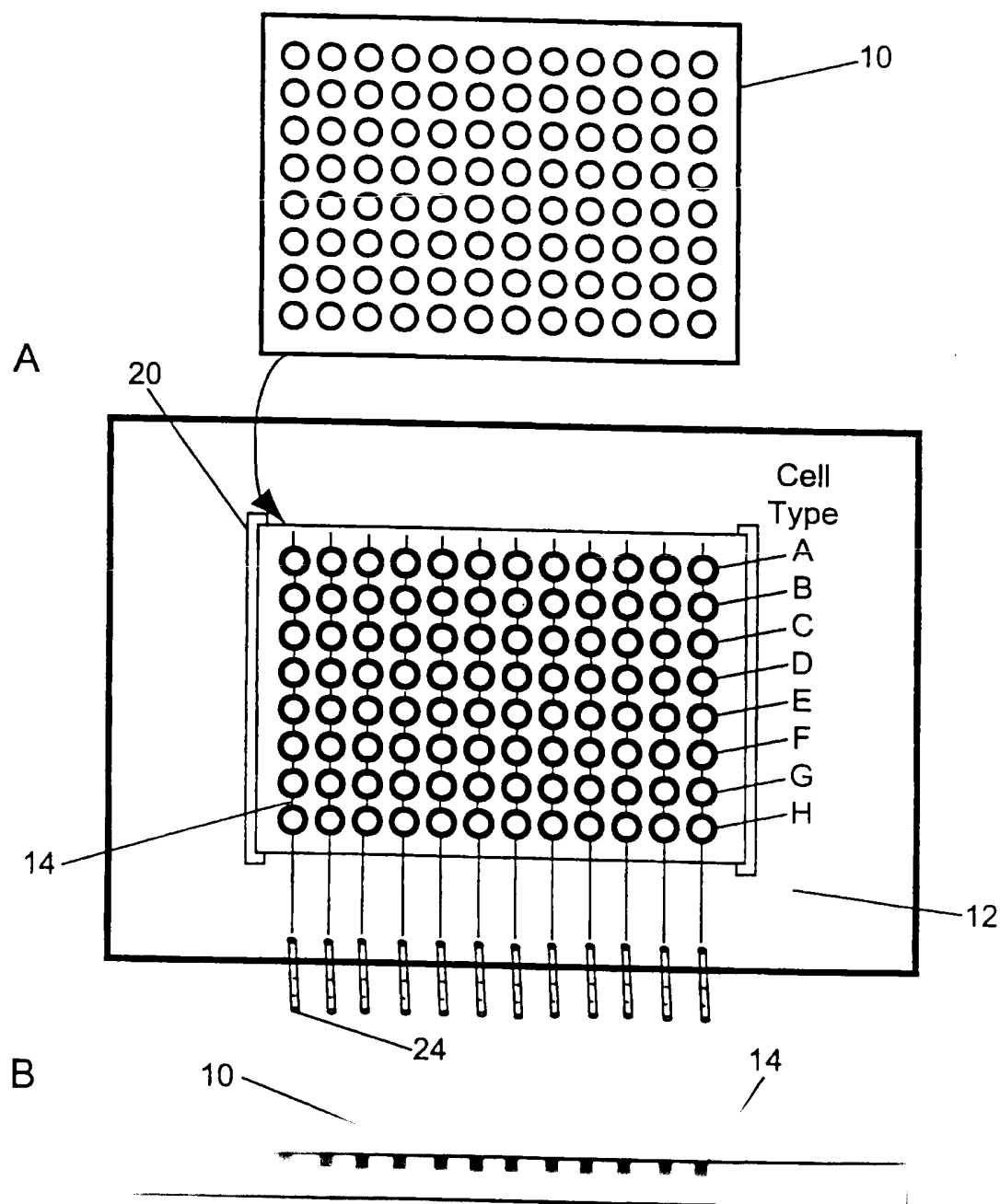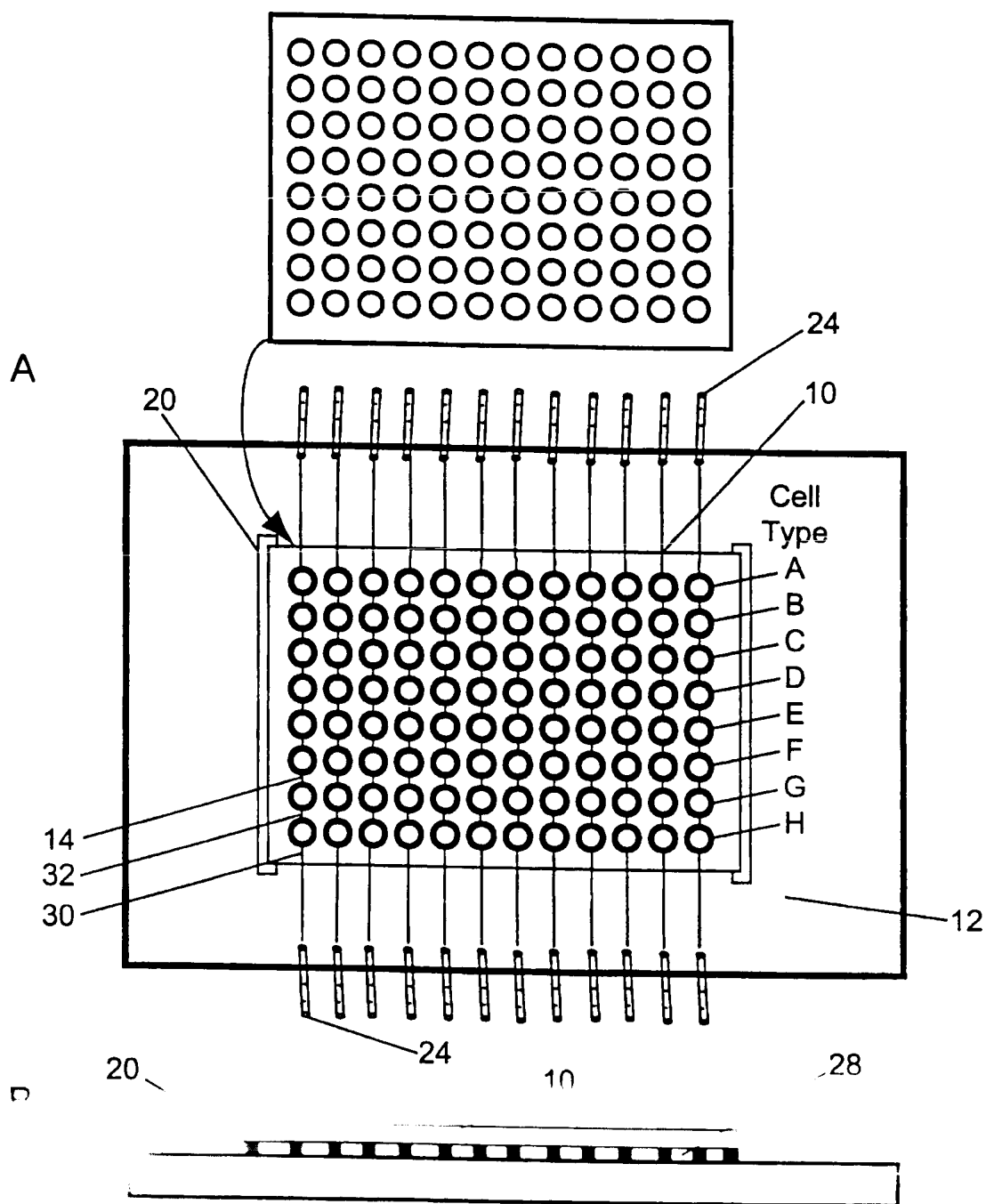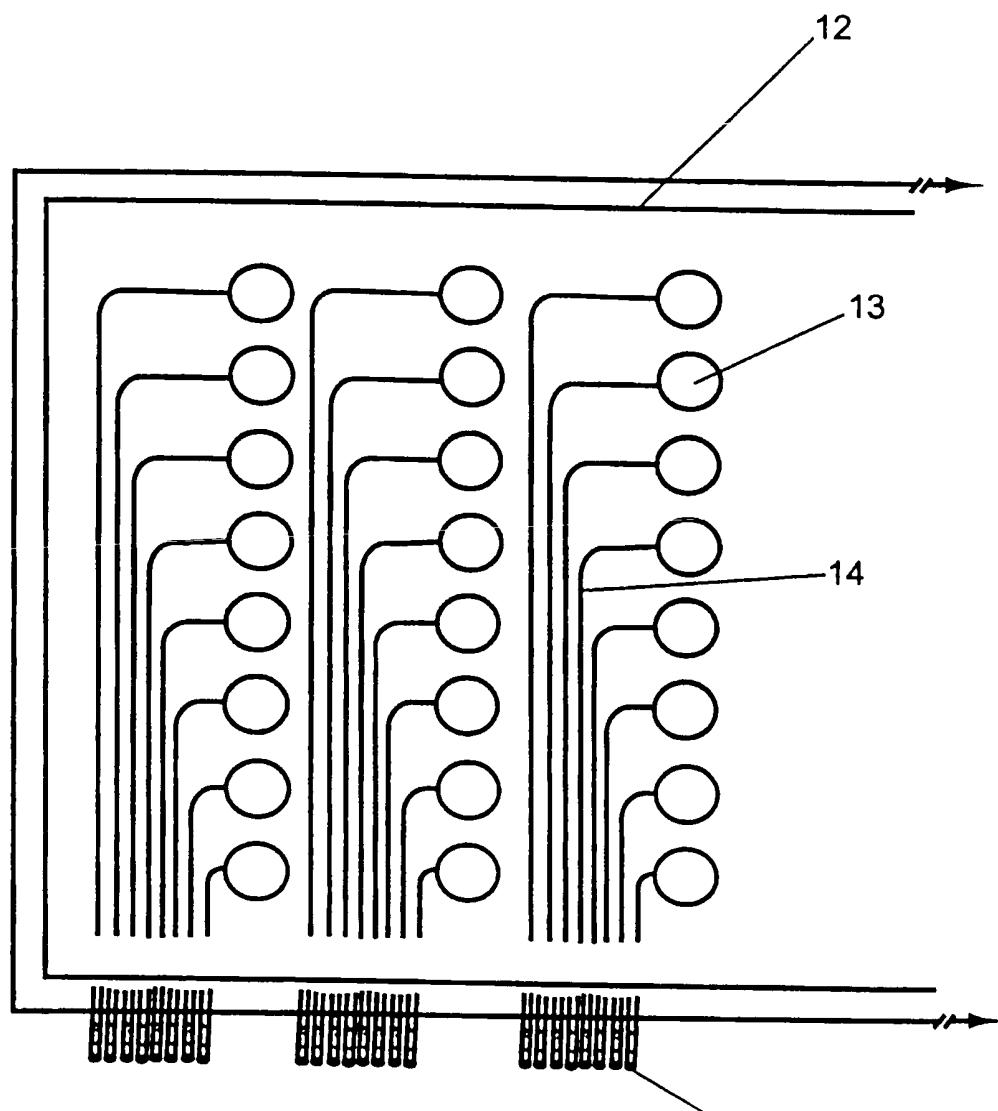
Figure 8

Figure 9

Figure 10
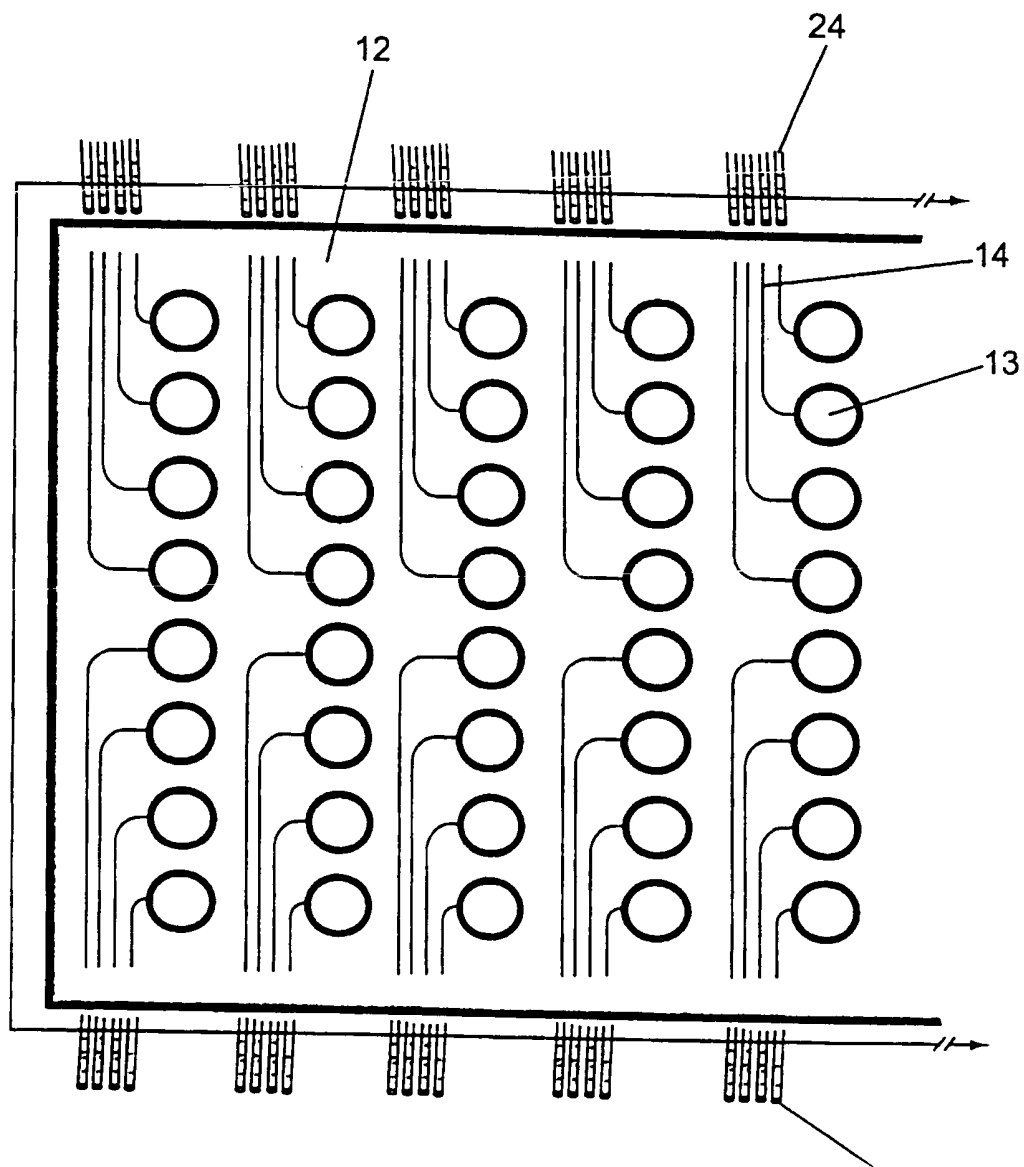
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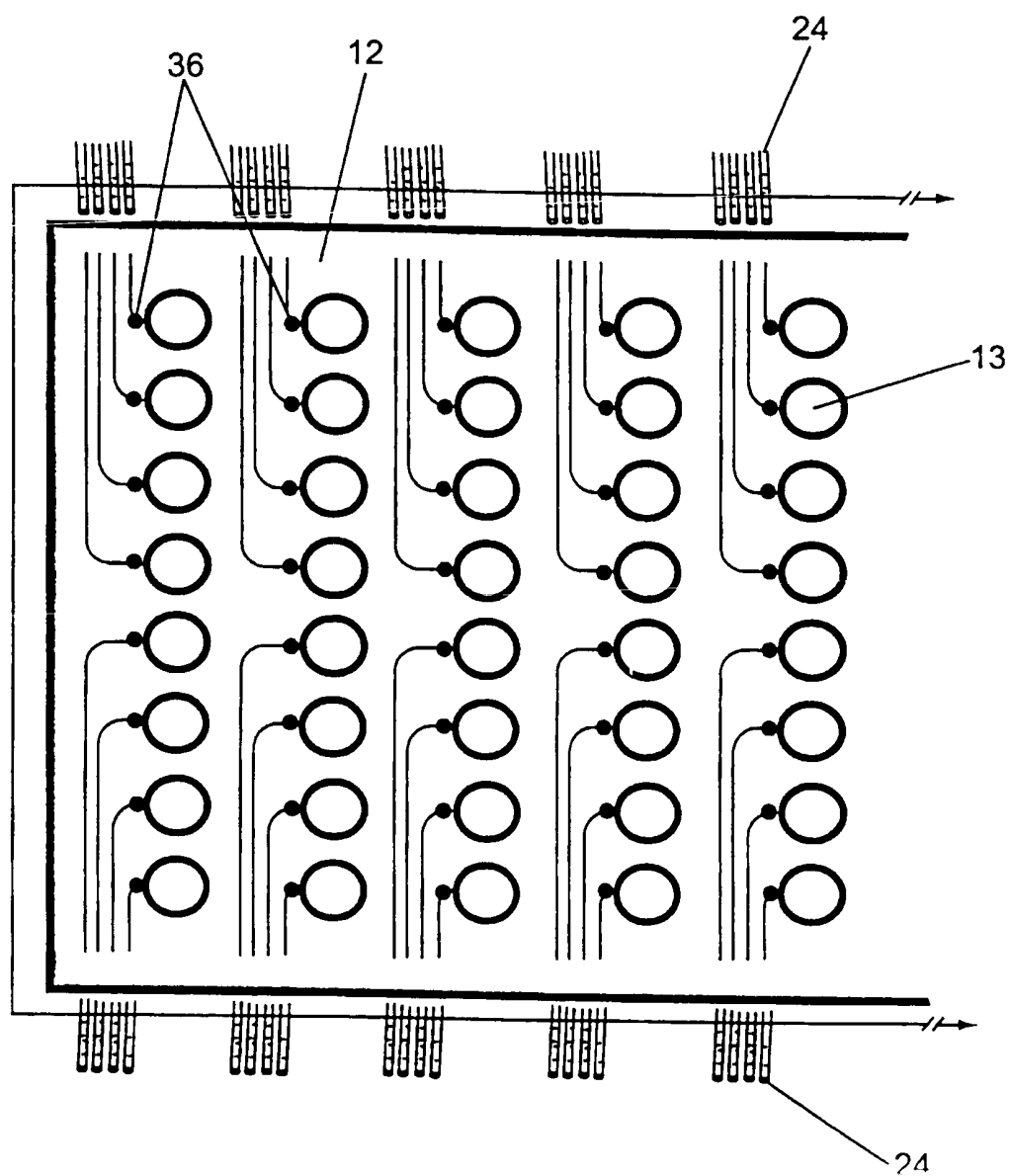
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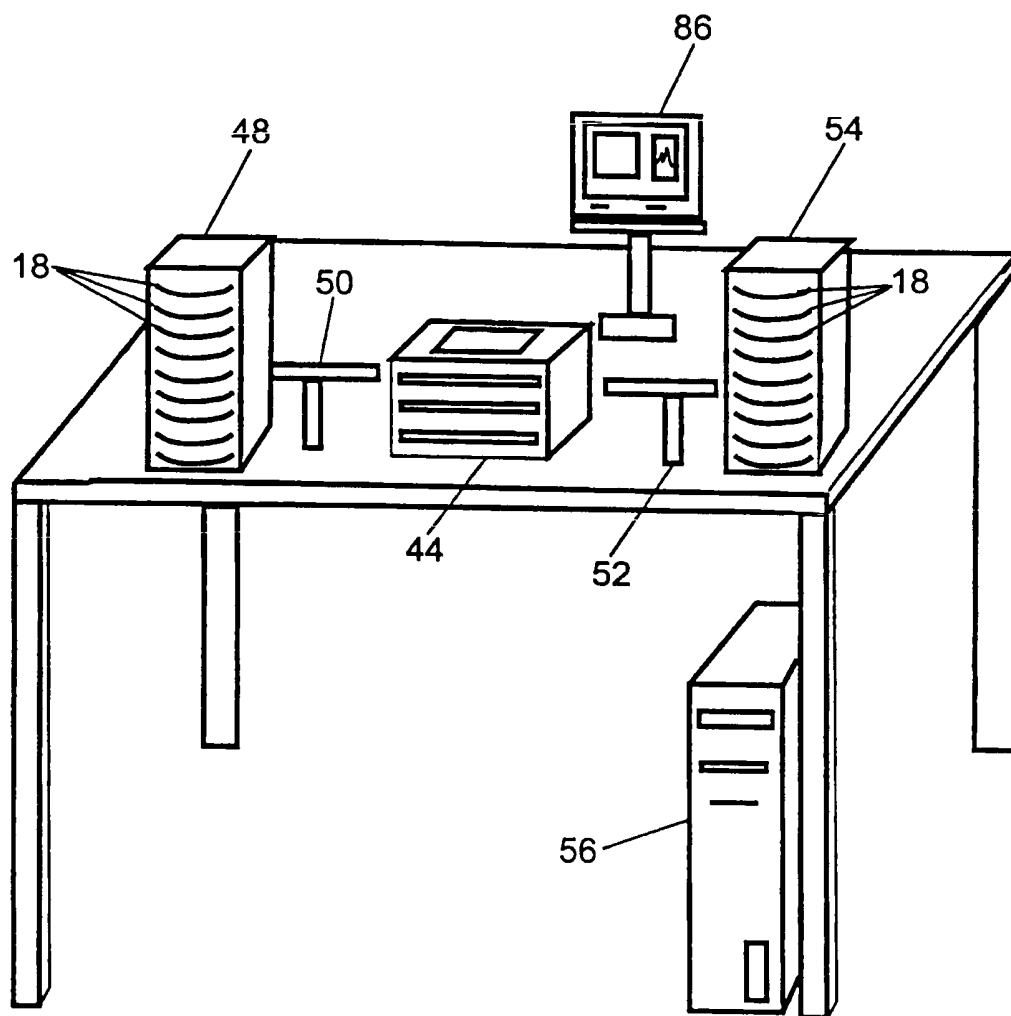
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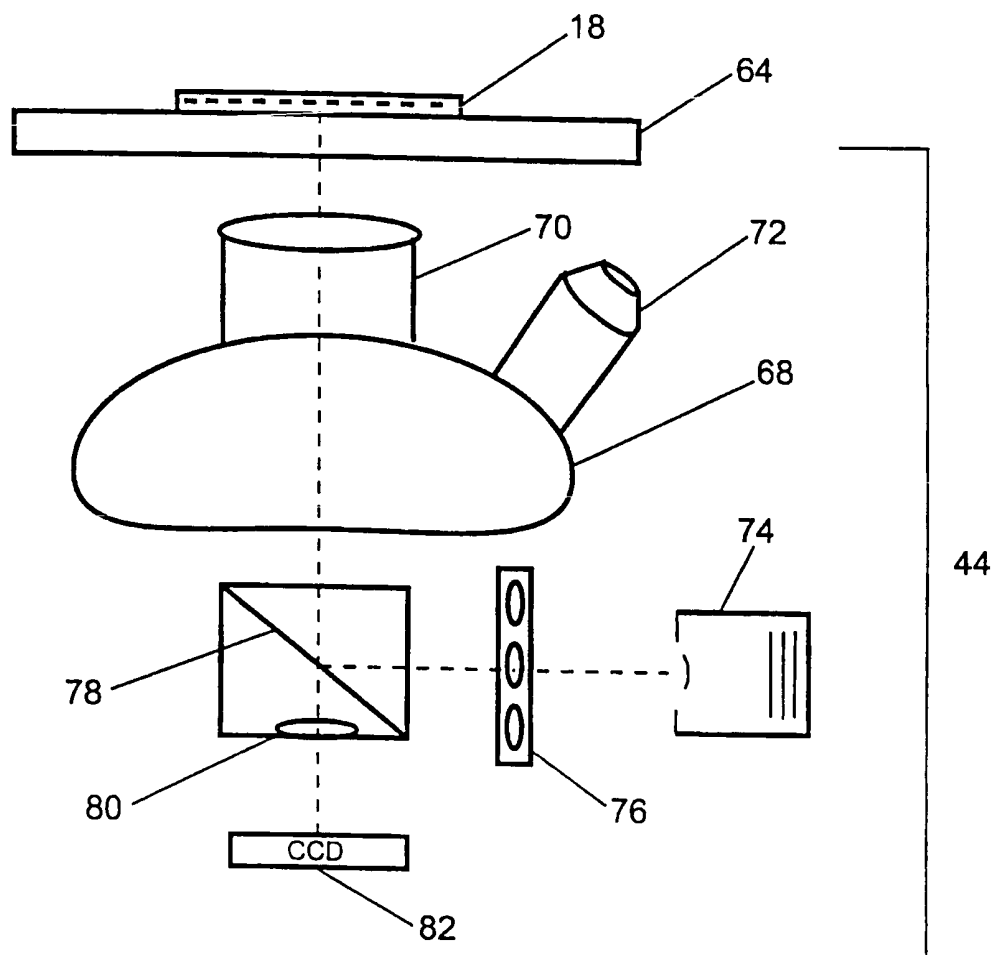
Figure 14A

Figure 14B

16/48

Figure 14C

17/48
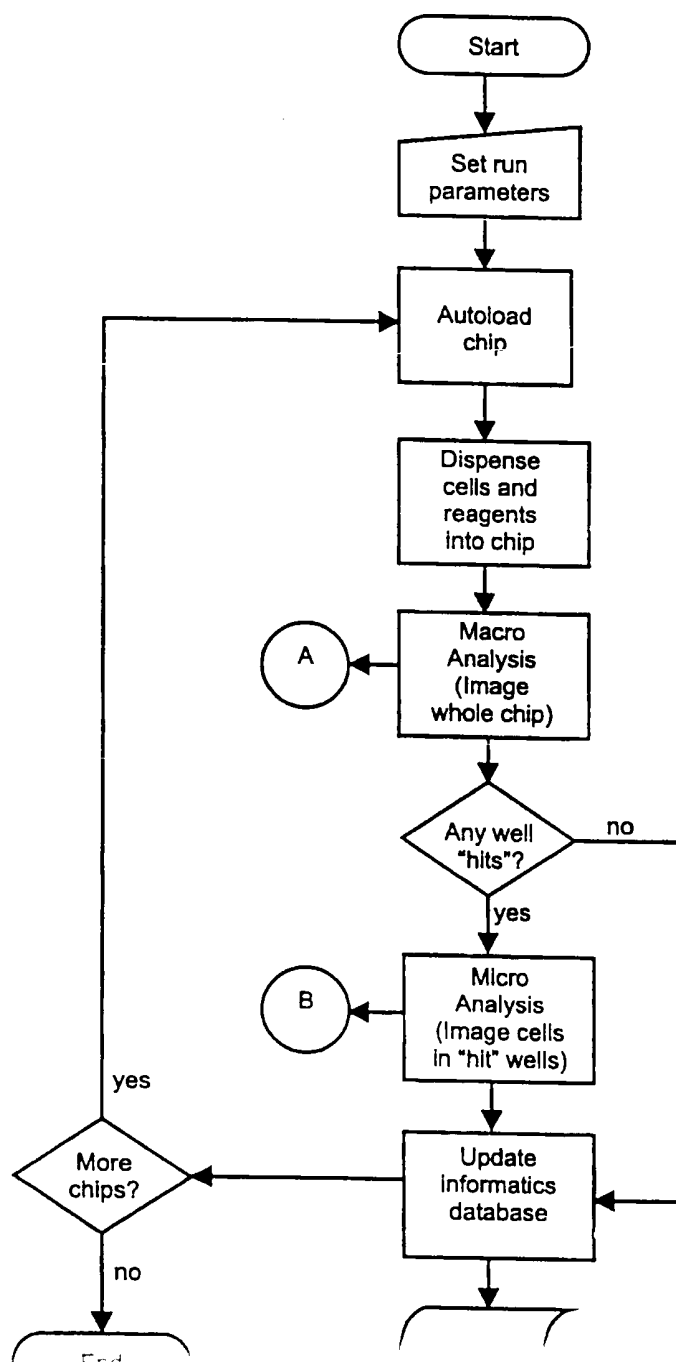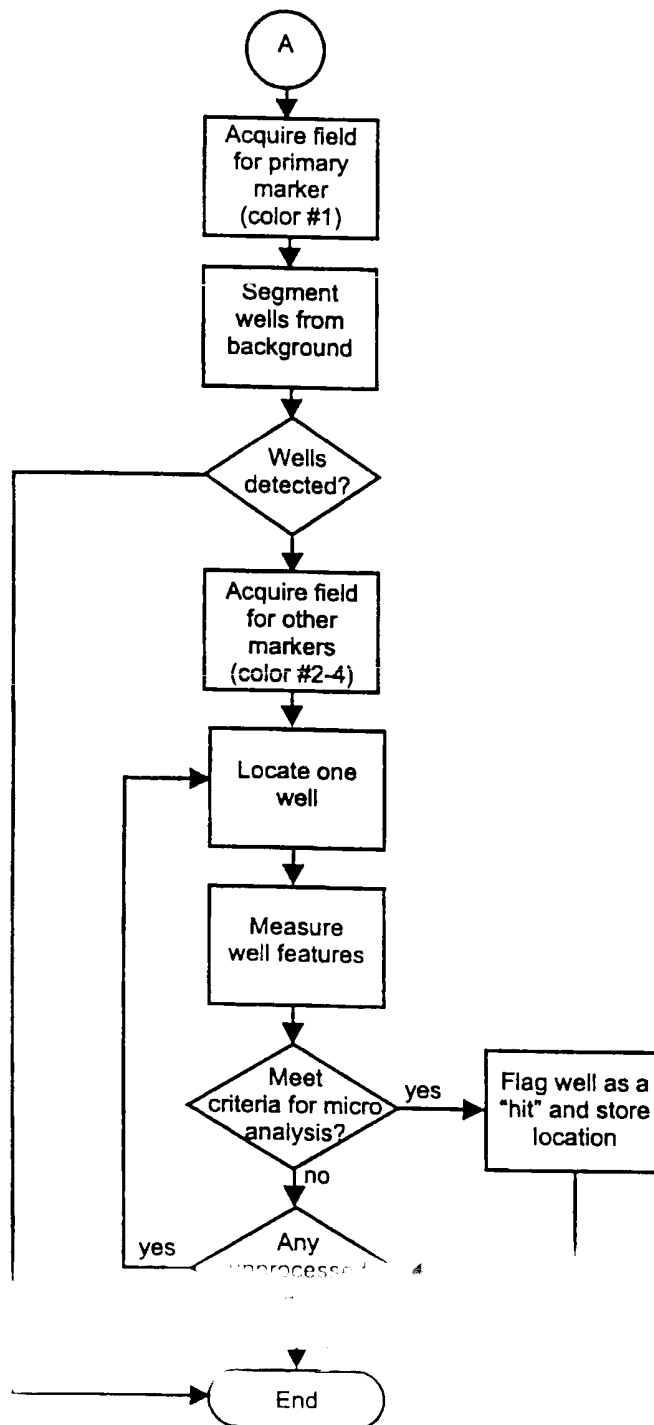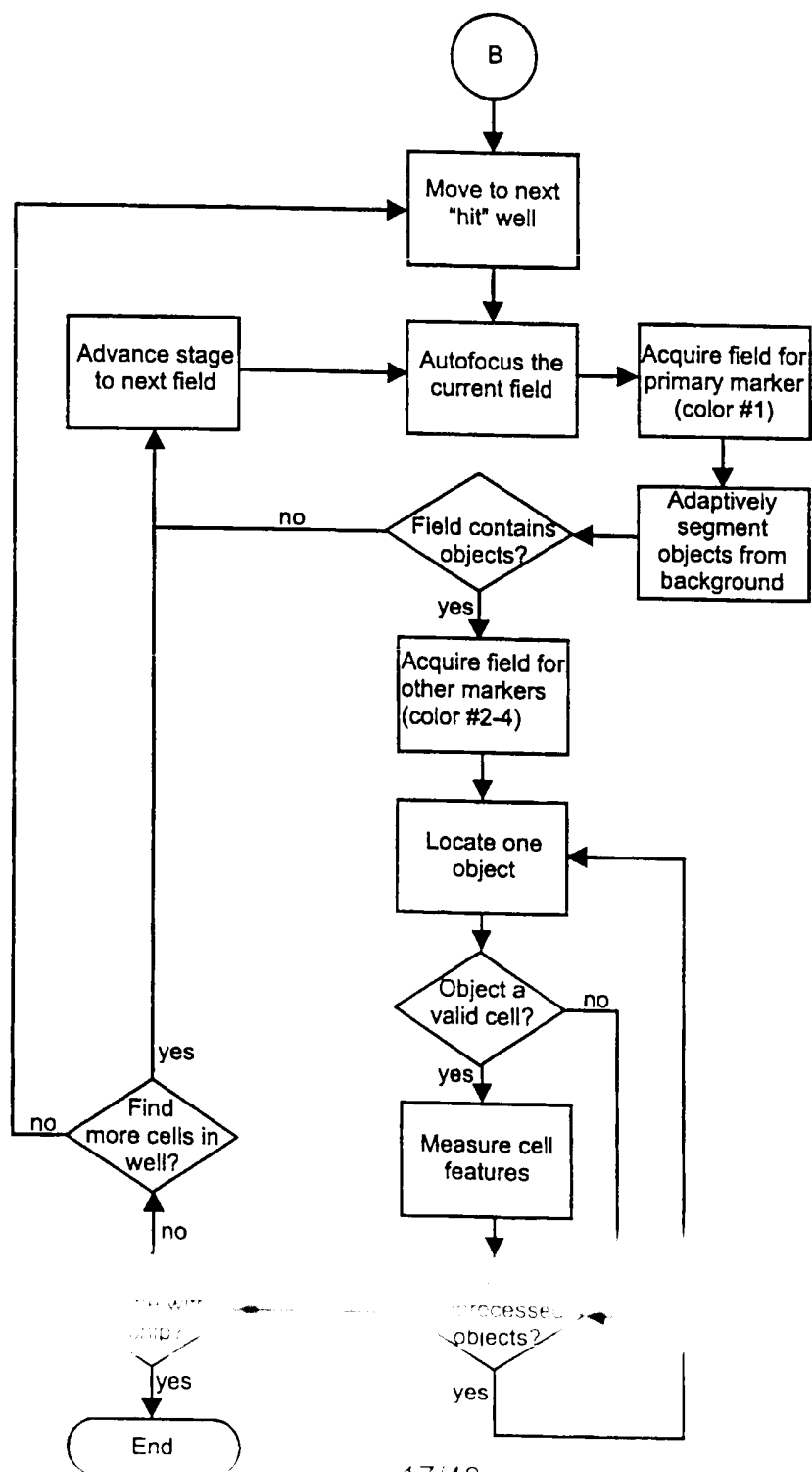
Figure 15

Figure 16

Figure 17

A

**Control
No Coating**

B

**IgM
Coated**

C

**Whole Serum
Coated**

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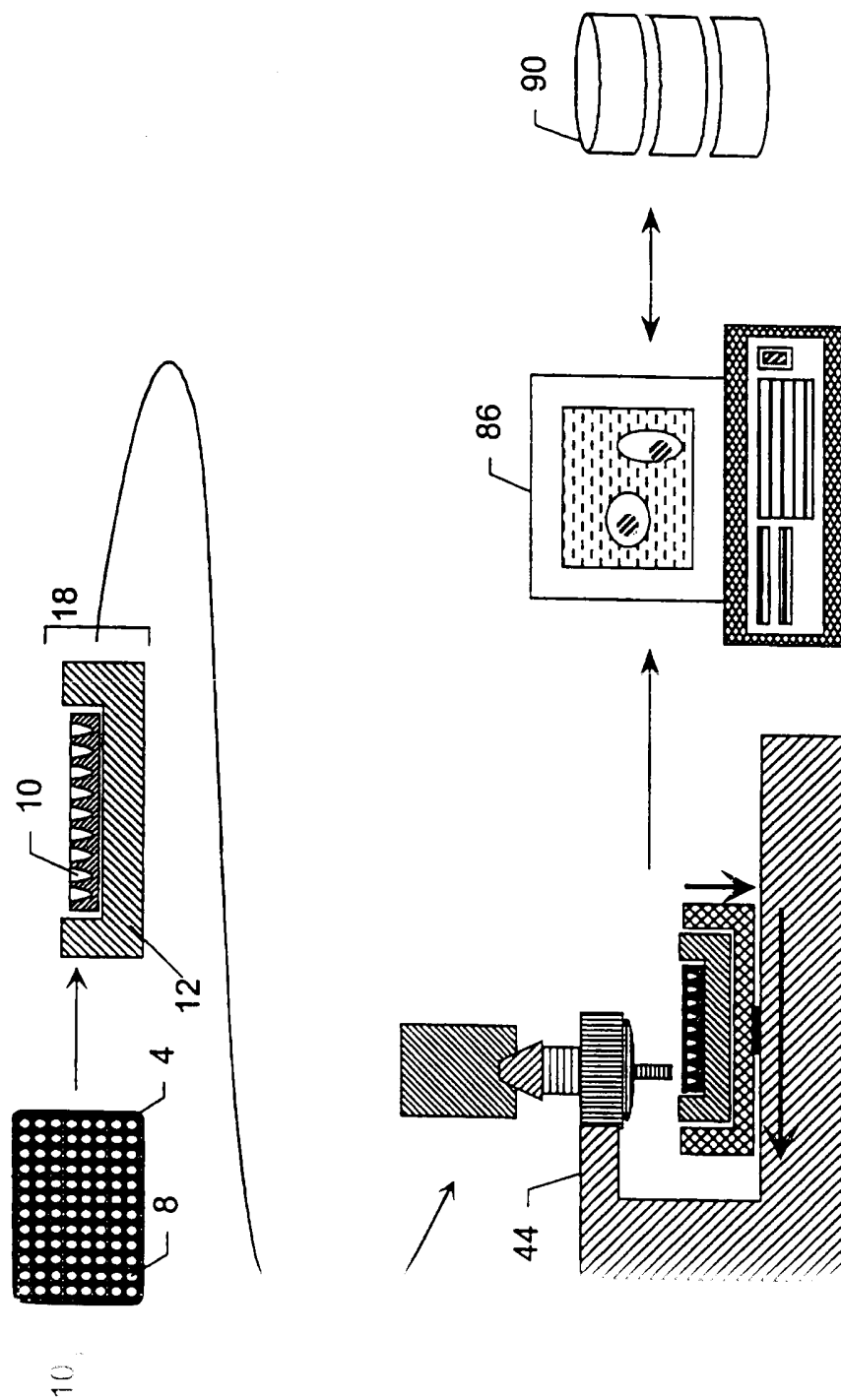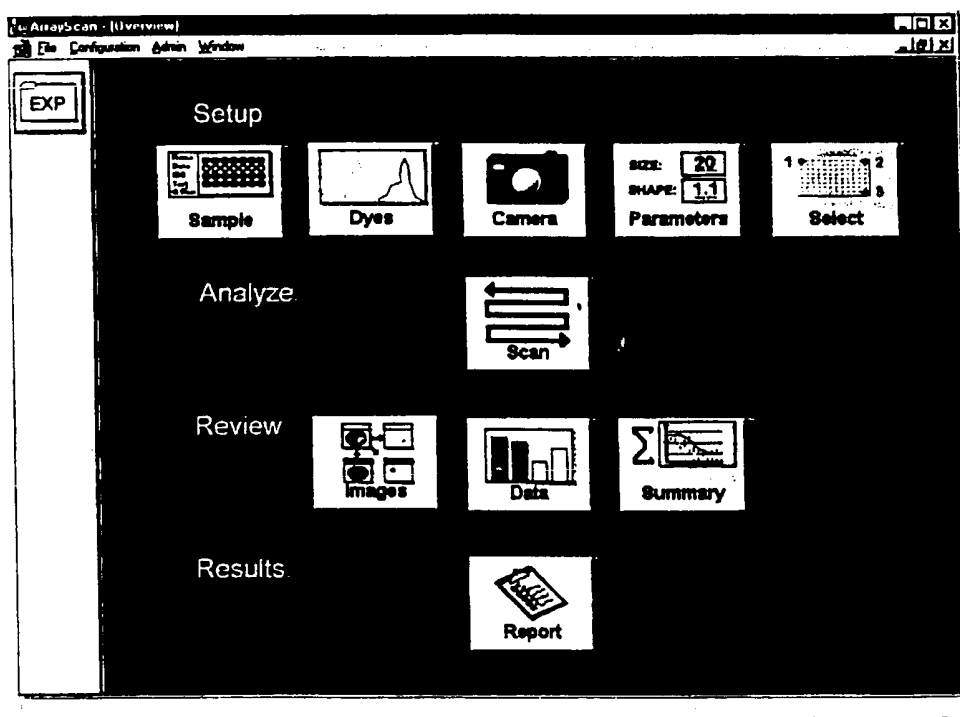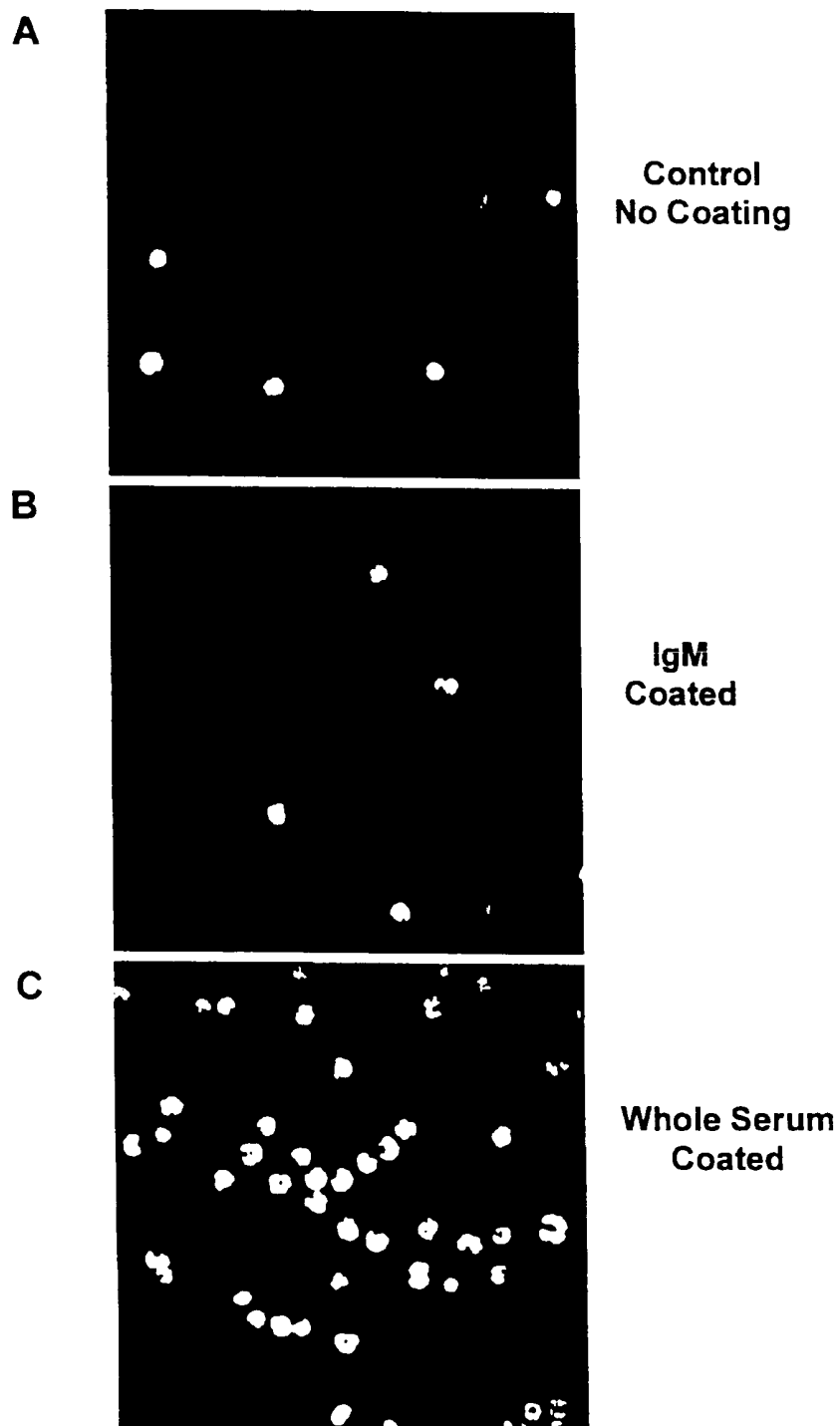
Figure 18

A

B

Figure 19

Figure 20

008   003

002

060

Figure 21

S U B S T I T U T E   S H E E T   (RULE 26)
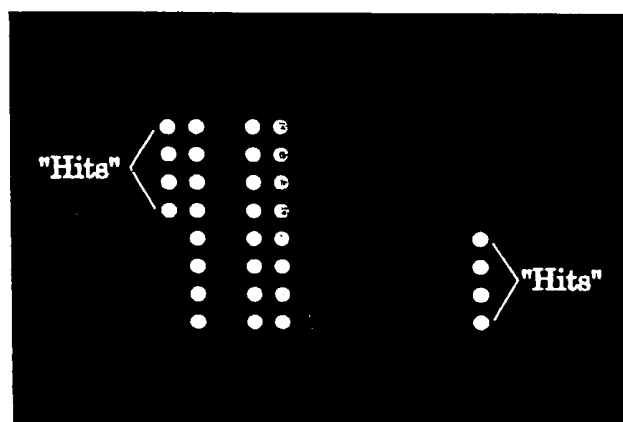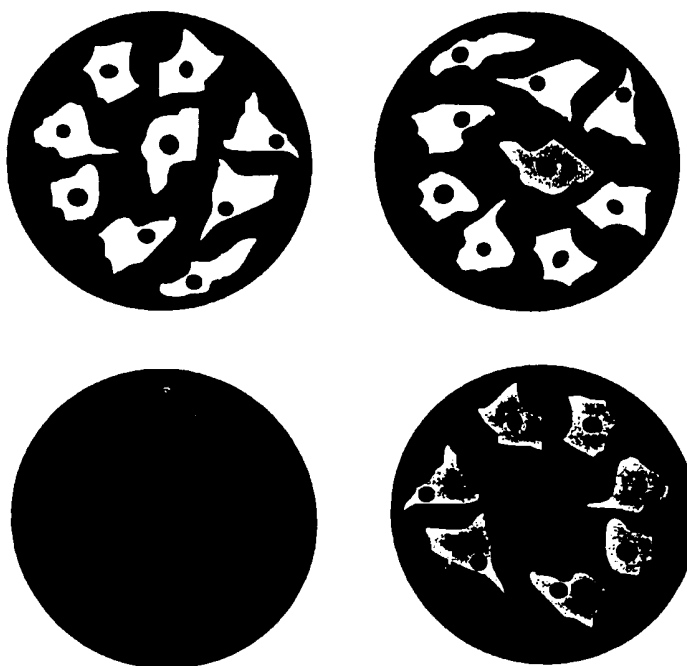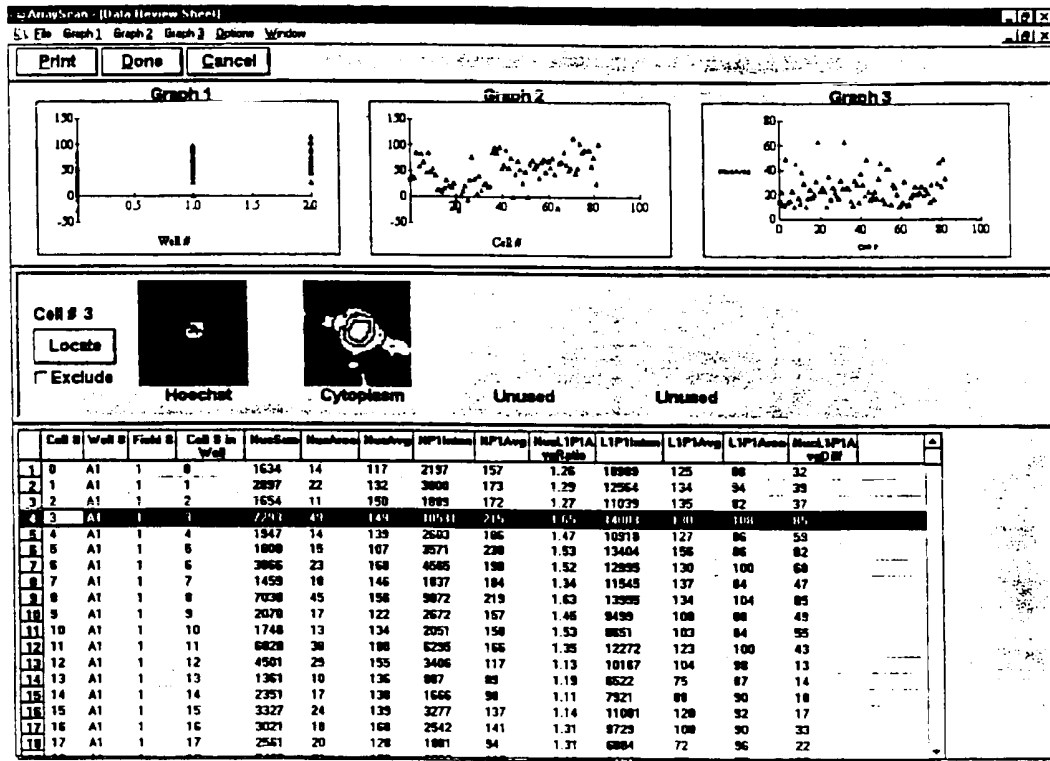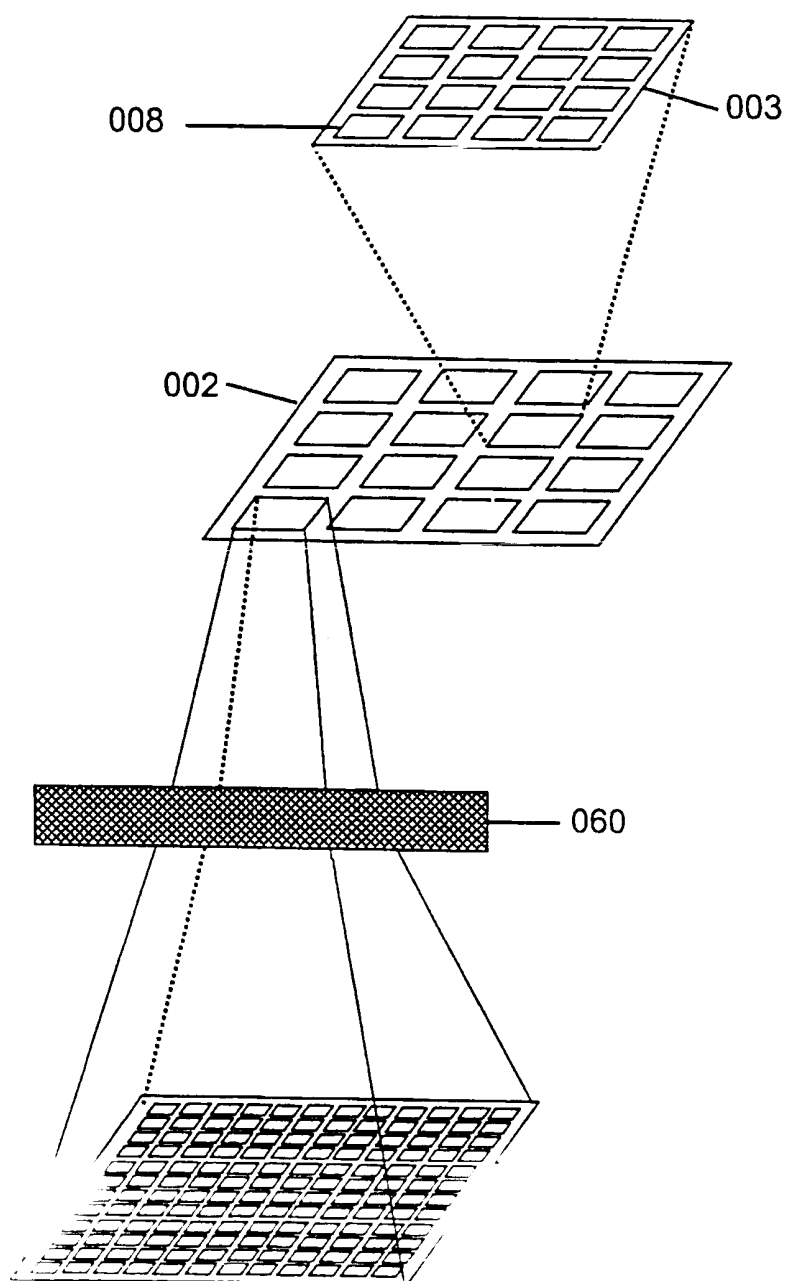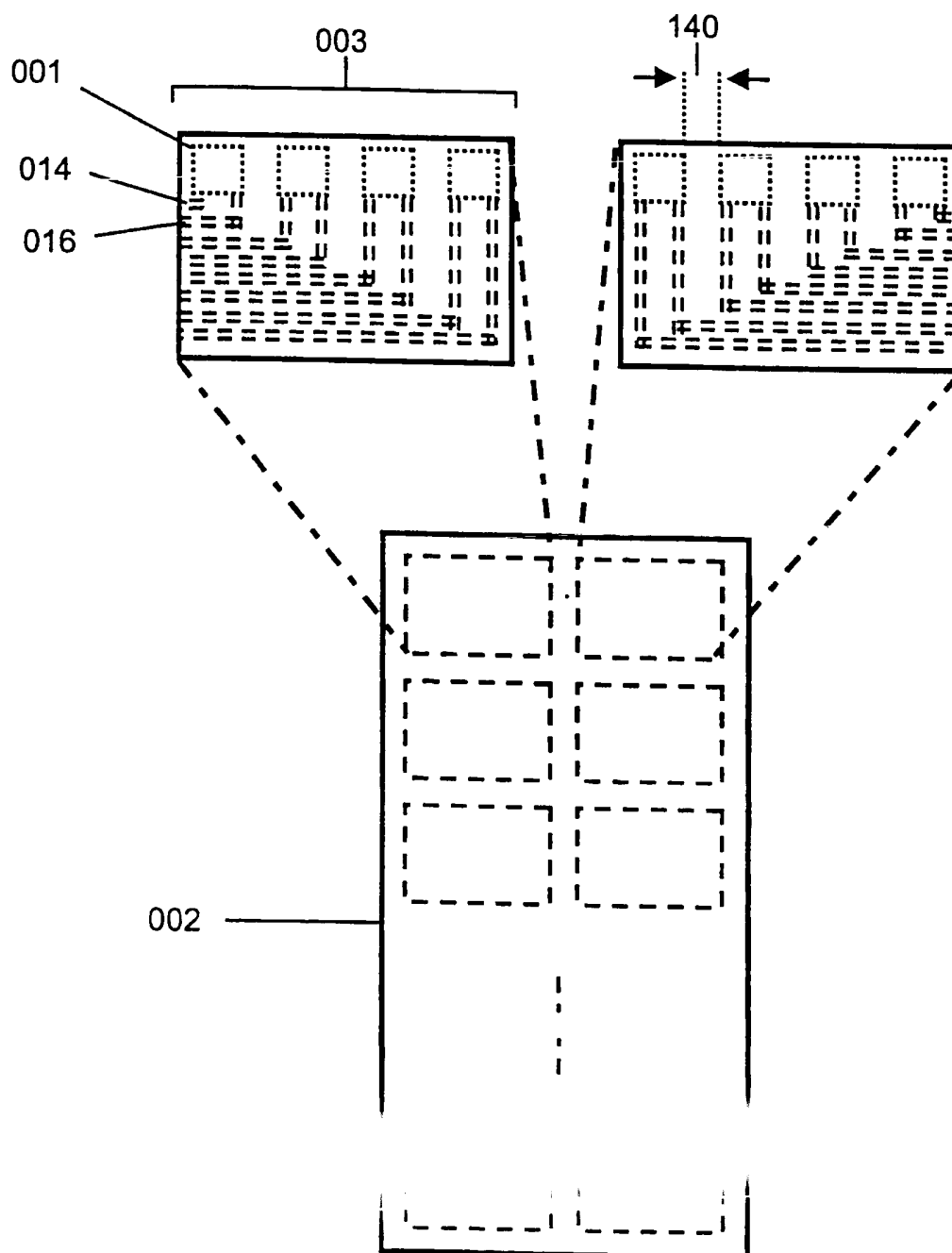
Figure 22

Figure 23
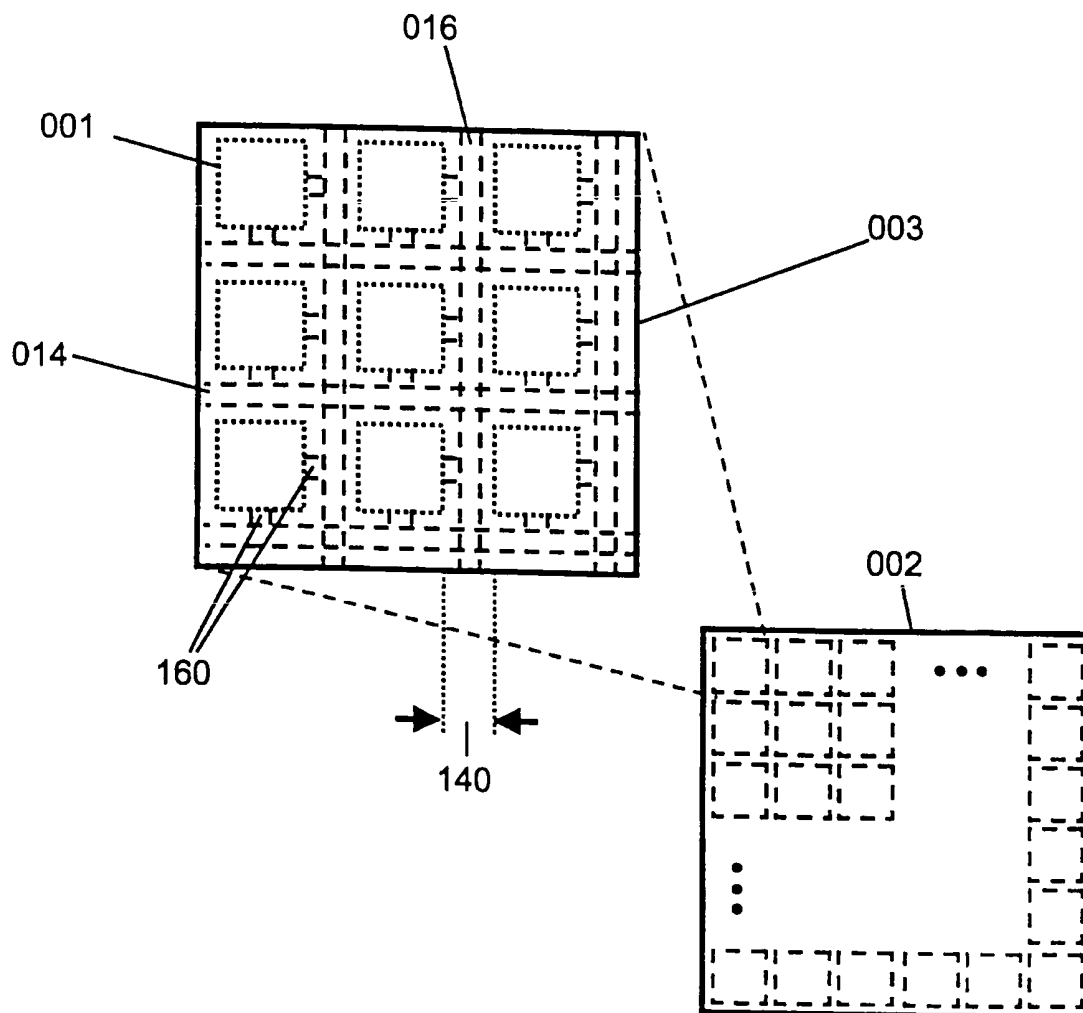
Figure 24
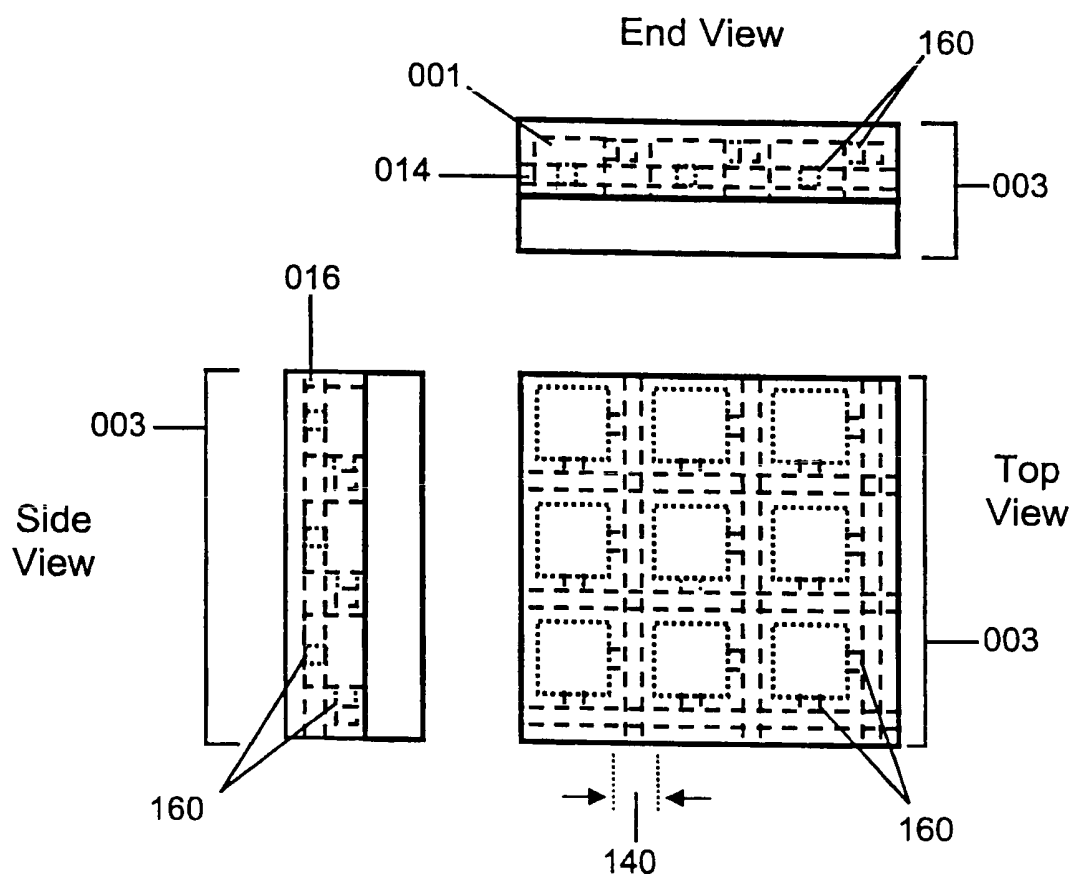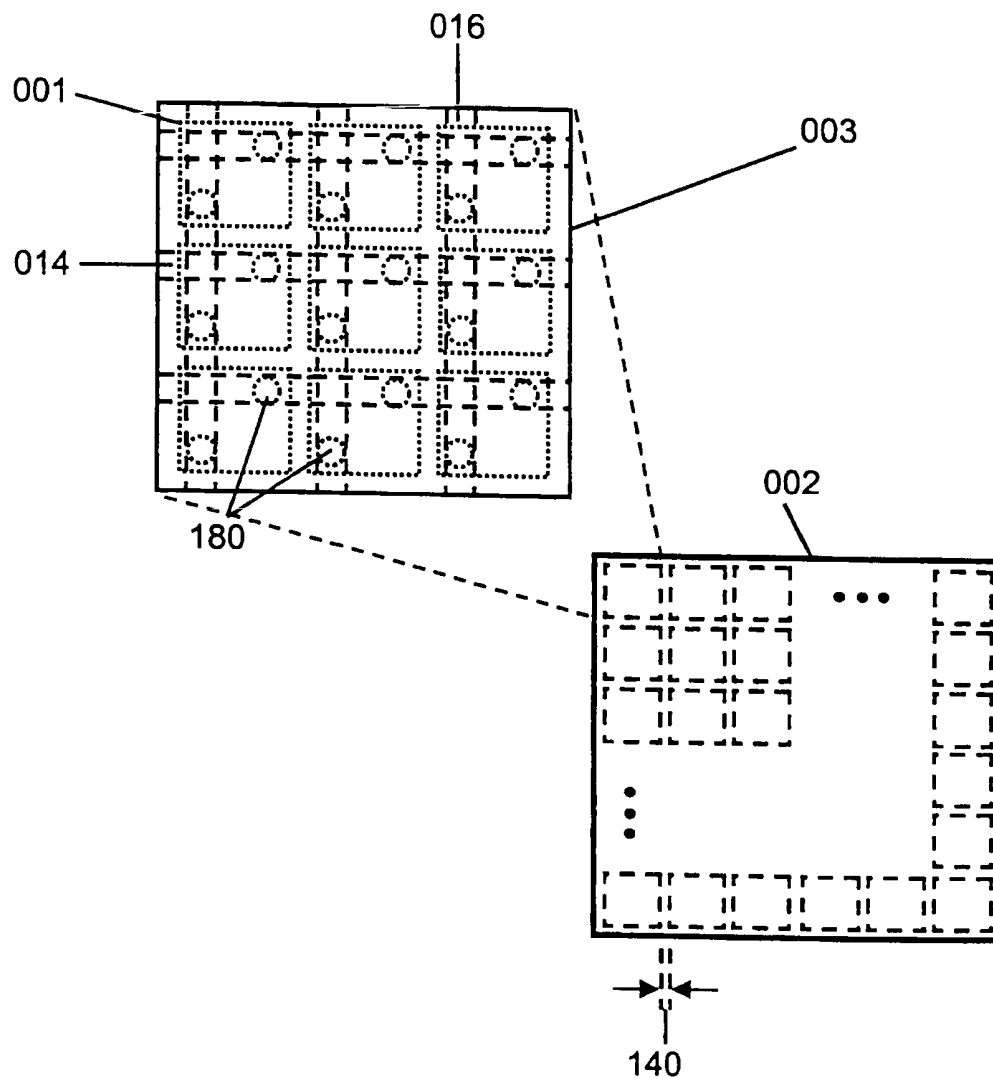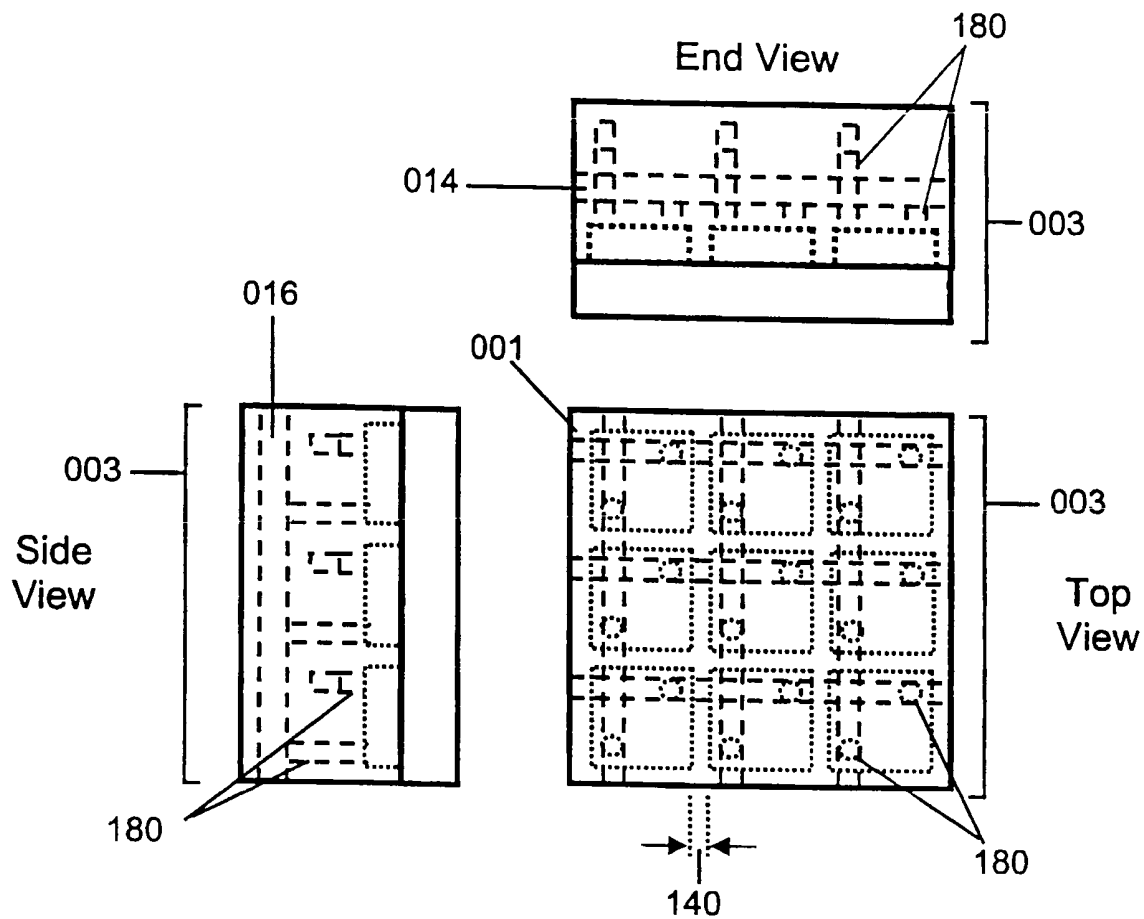
End View

Side View

Top View

Figure 25

Figure 26

End View

180

014

003

016

003

Side
View

001

003

Top
View

180

180

140

180

Figure 27

Figure 28

SUBSTITUTE SHEET (RULE 26)

Figure 29

Figure 30

SUBSTITUTE SHEET (RULE 26)

Figure 31

Figure 32

SUBSTITUTE SHEET (RULE 26)

Figure 33

Figure 34

End View

130

180

014

001

003

1   2   3

016

003

Side
View

180

Top
View

003

180

140
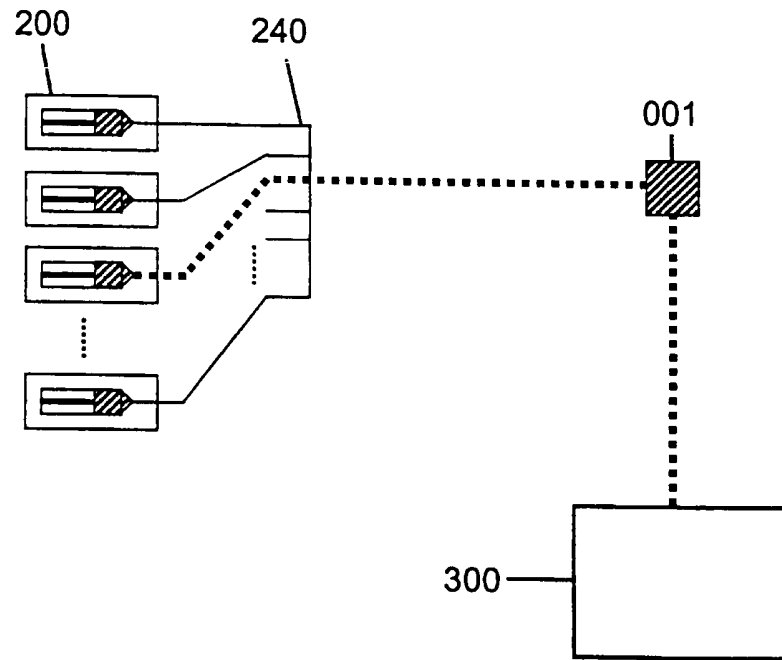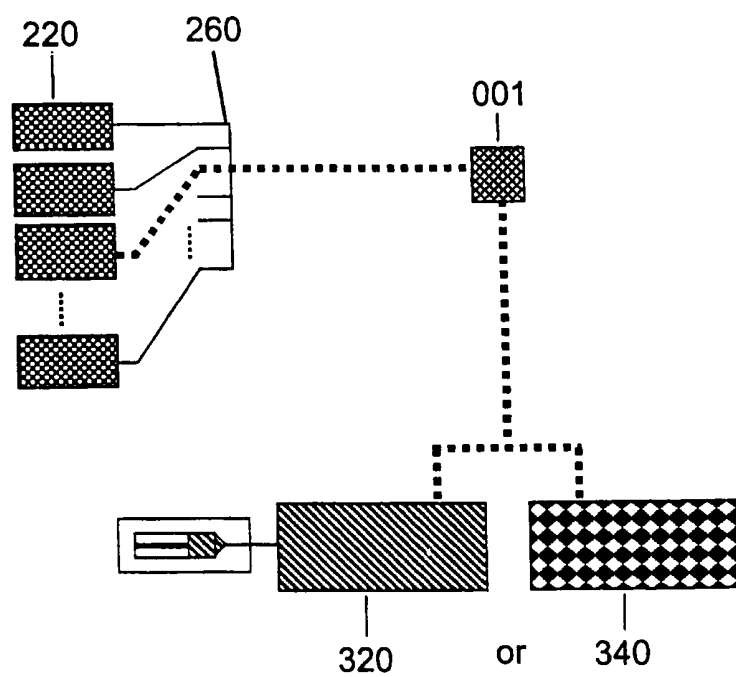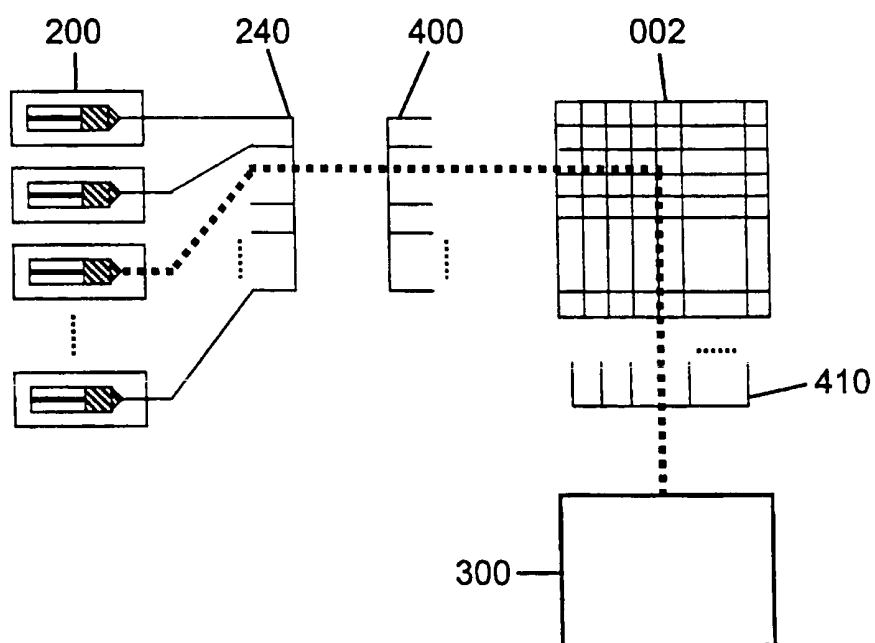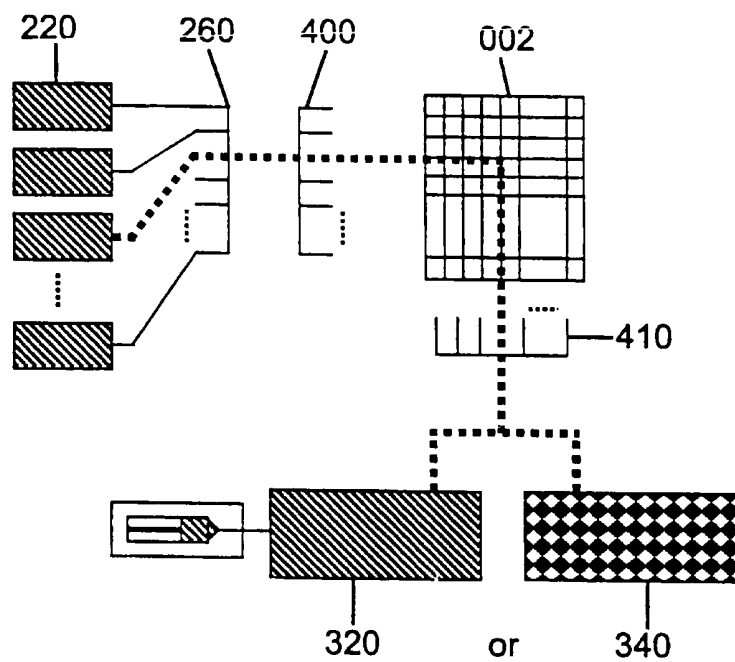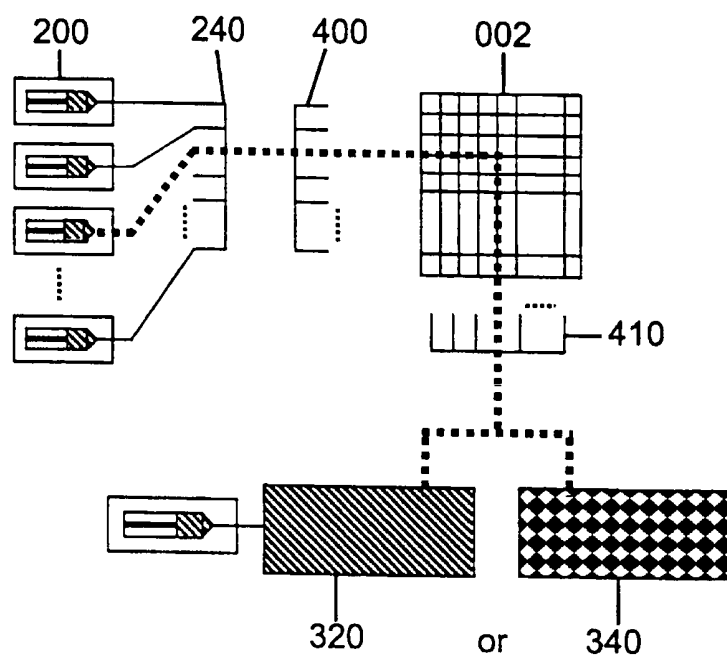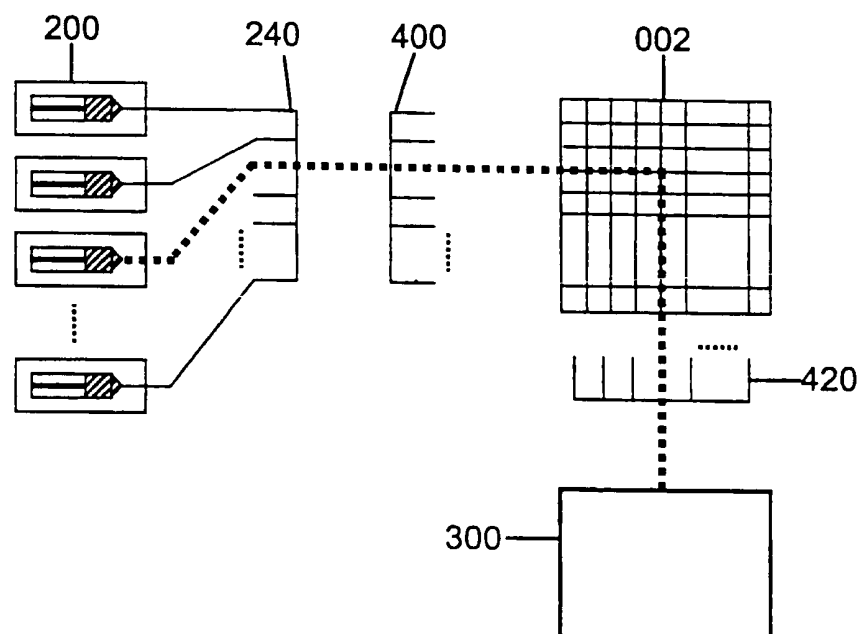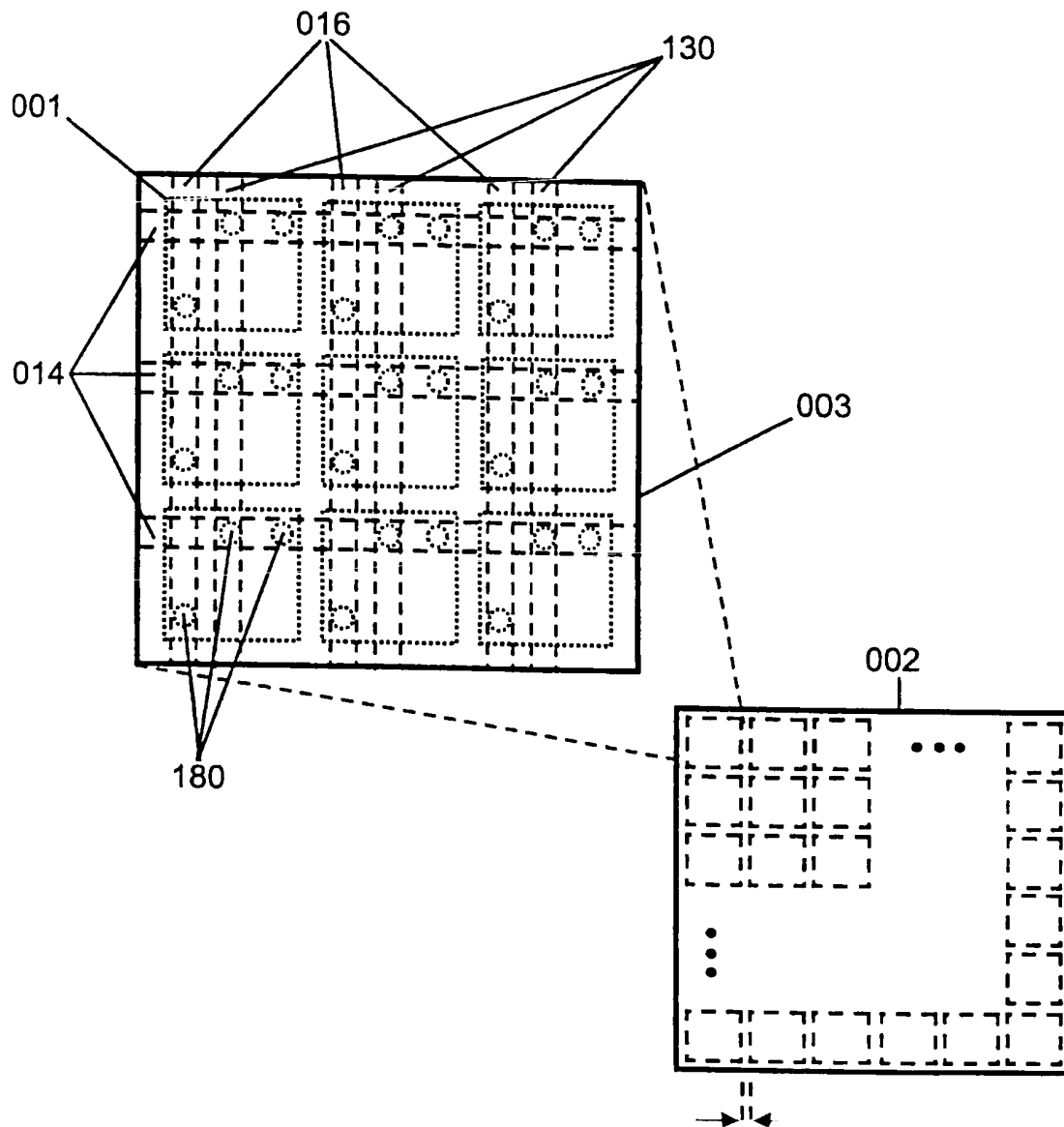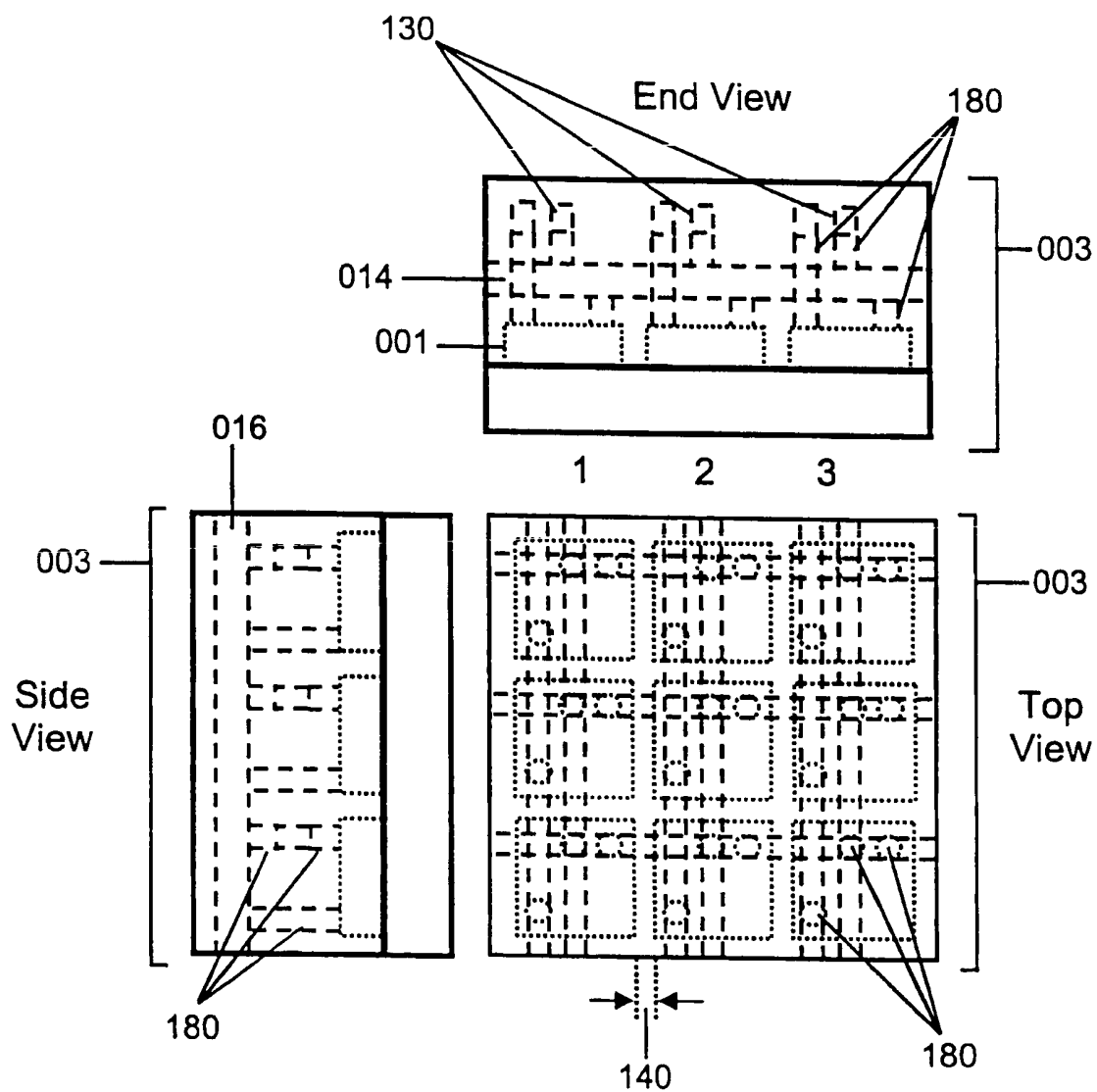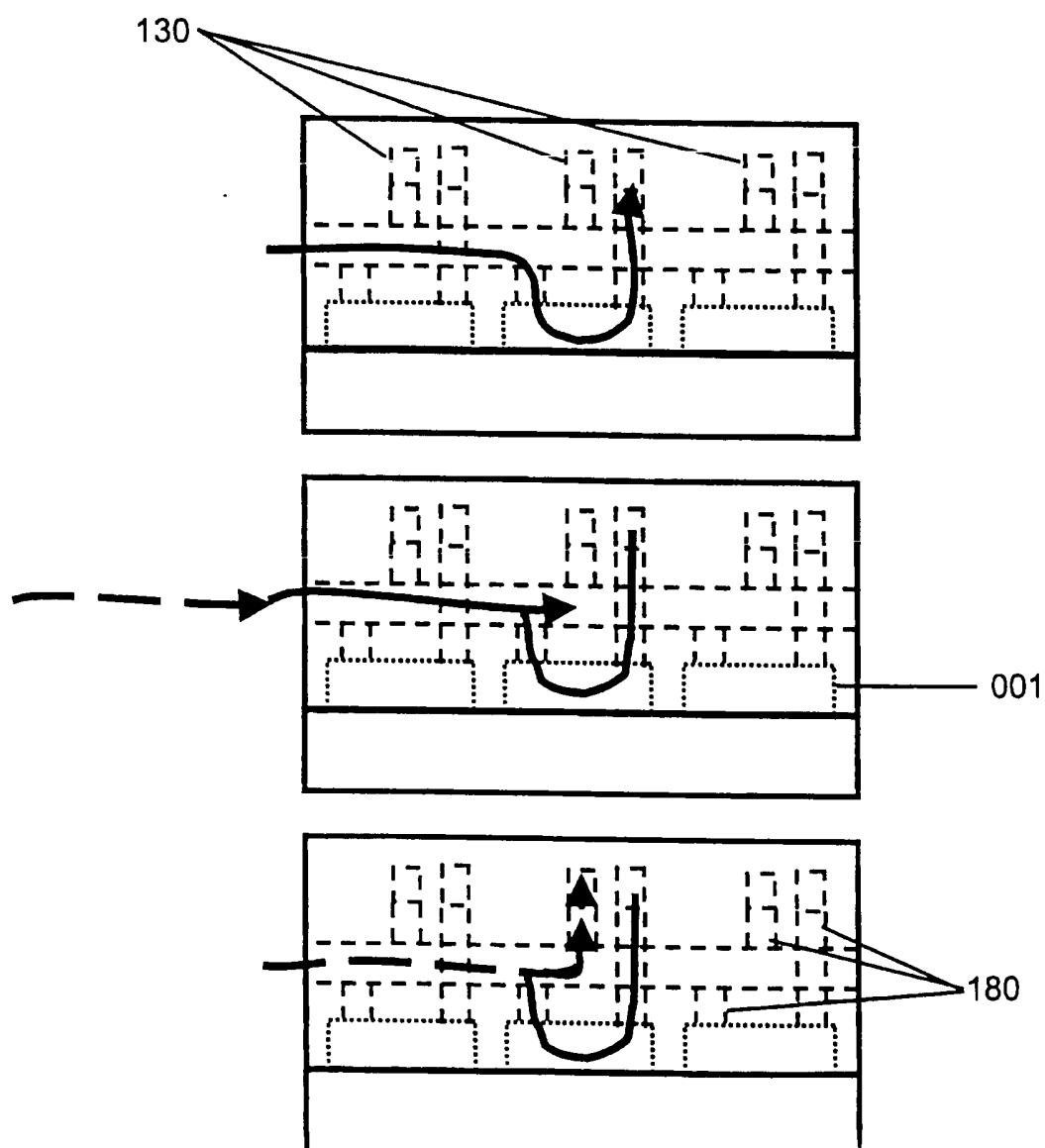
Figure 35

Figure 36

Figure 37

Figure 38

540

Figure 39

○ open valve
⊗ closed valve

End View

014

560

016

580

Side
View

Top
View

580

580
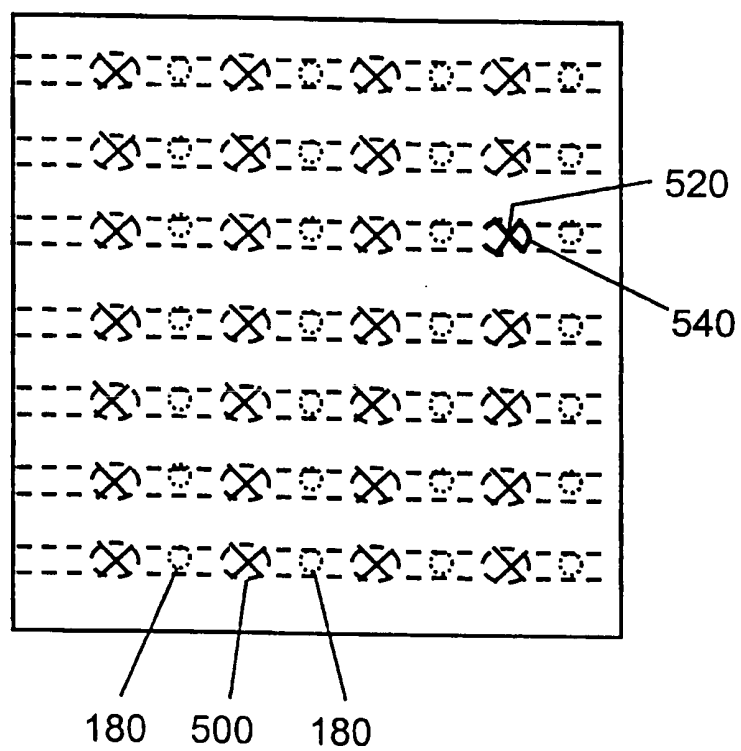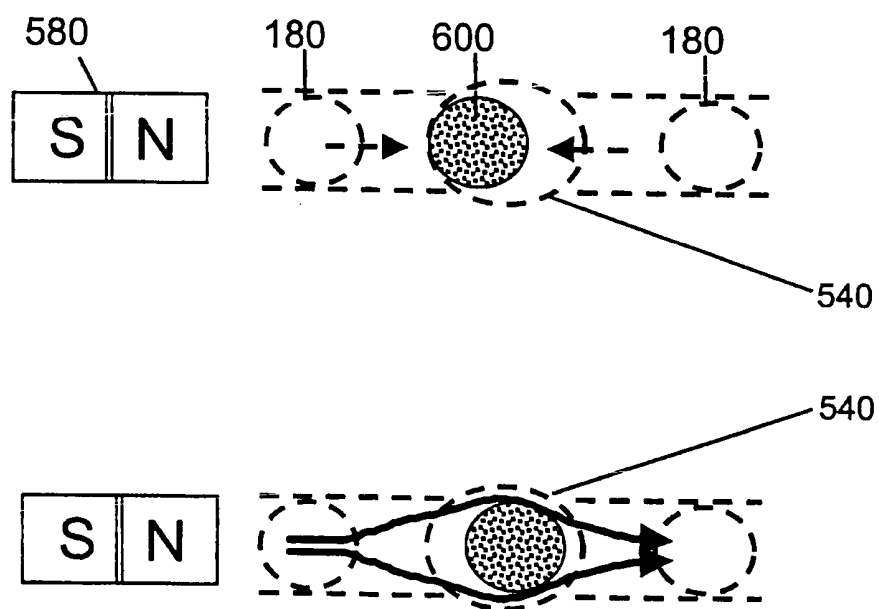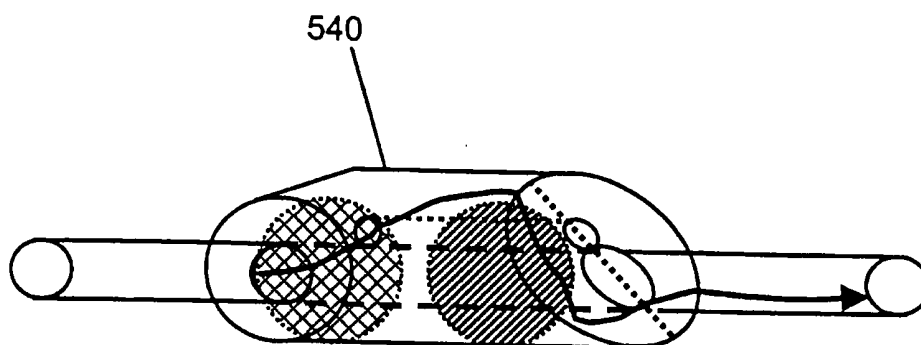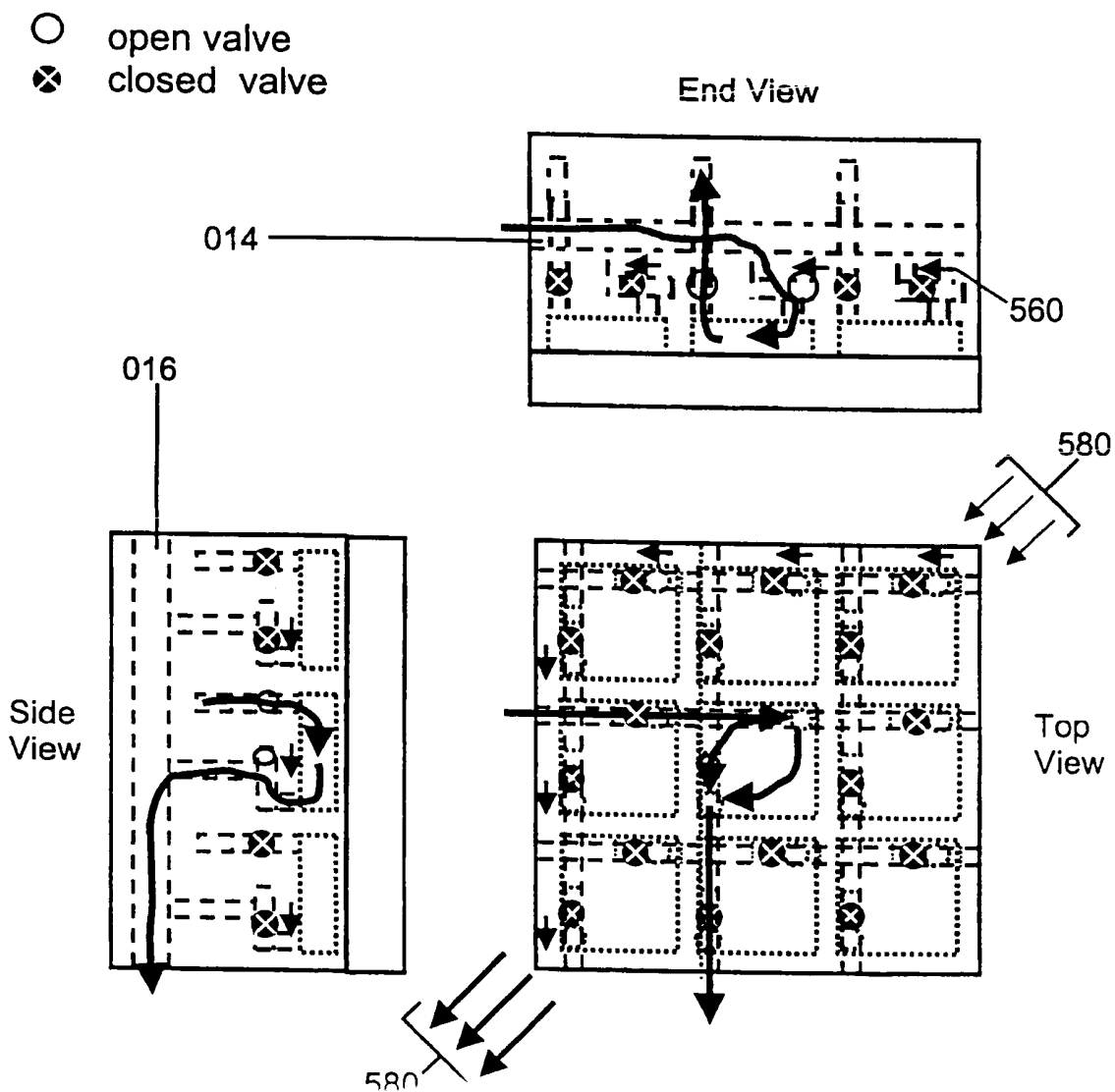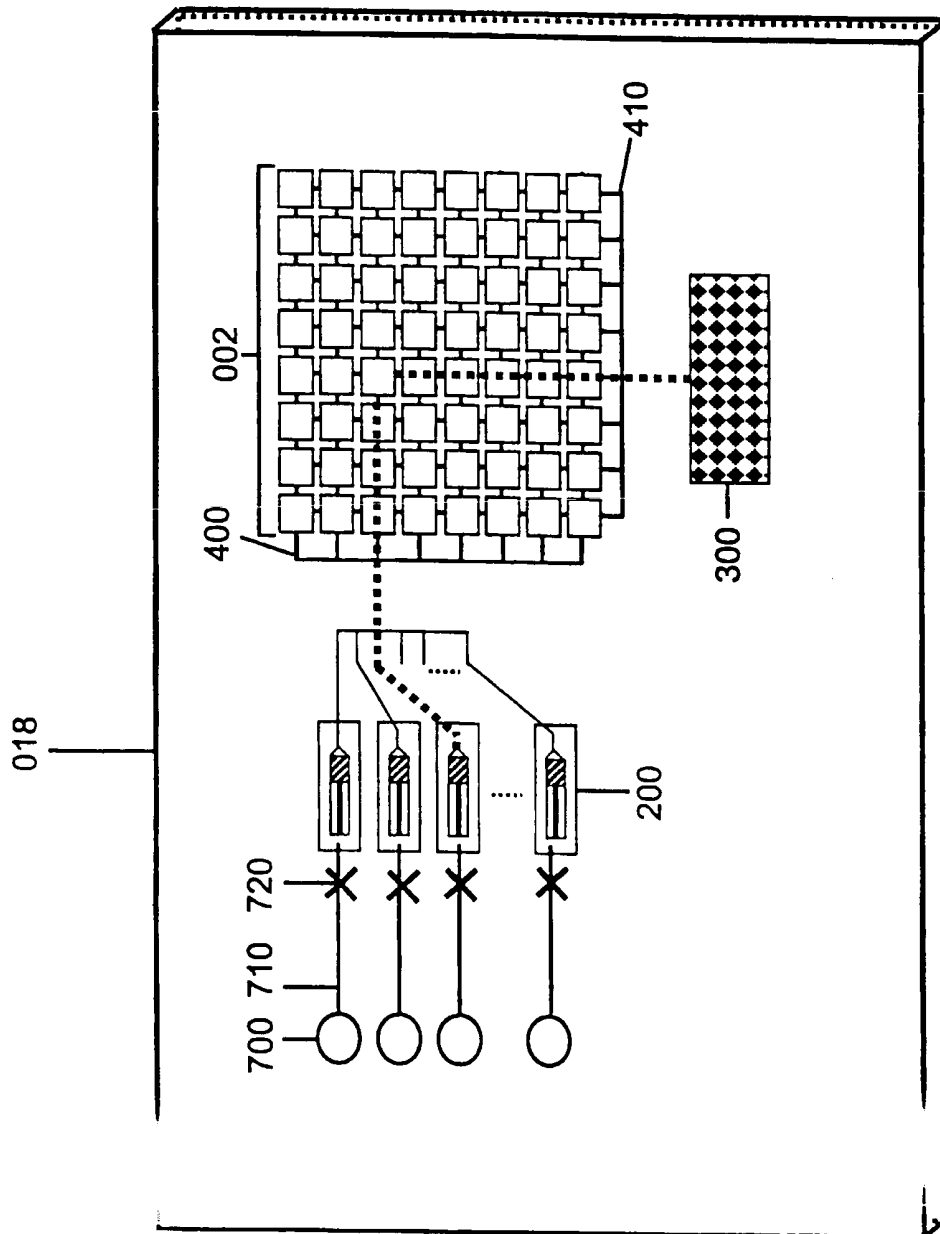
Figure 40
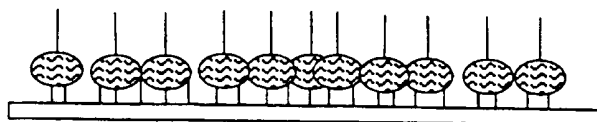
Figure 41
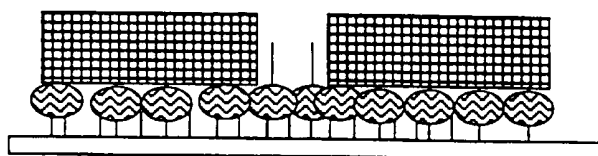
Figure 42

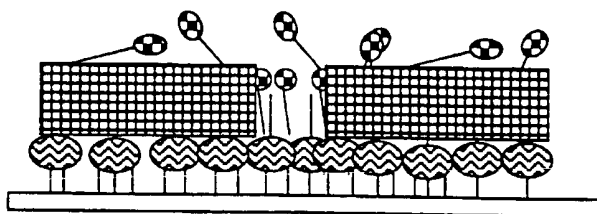
A

B

45/48
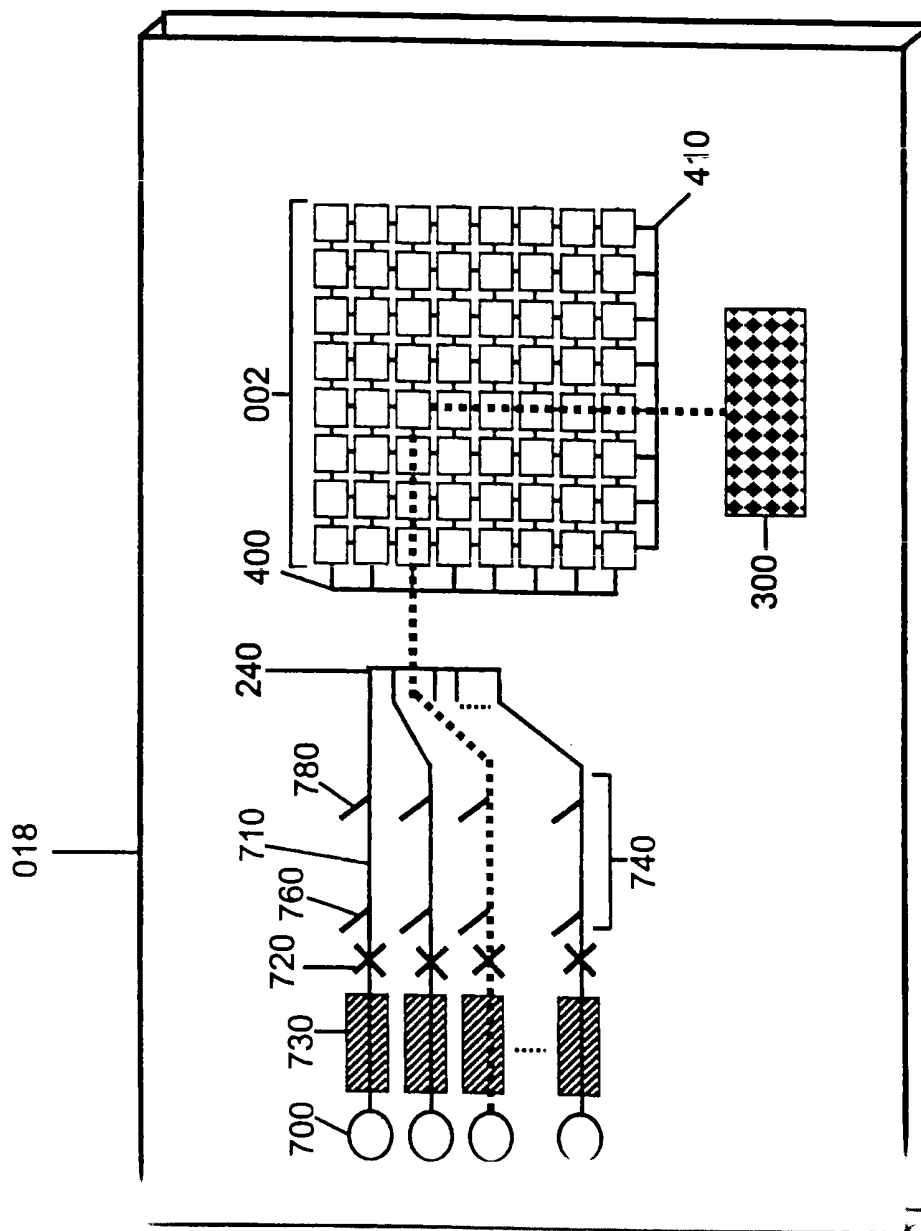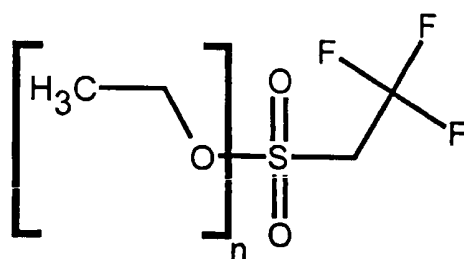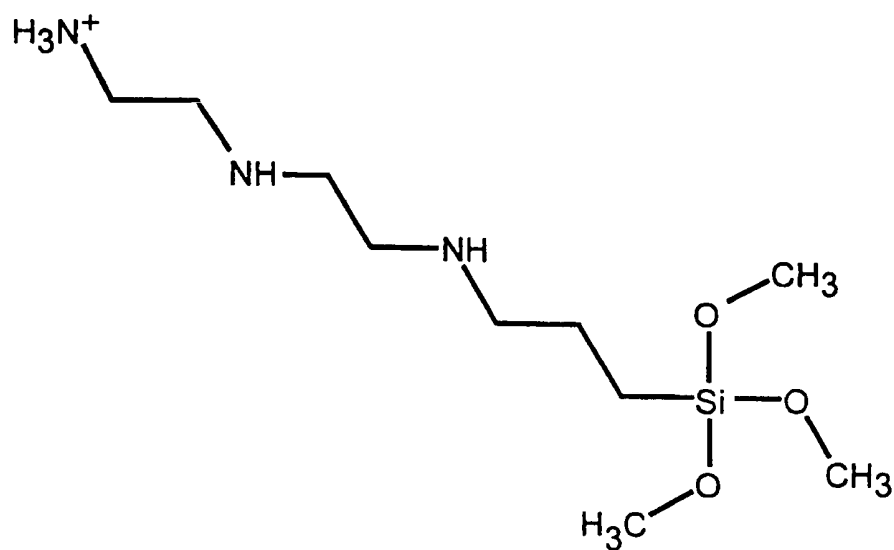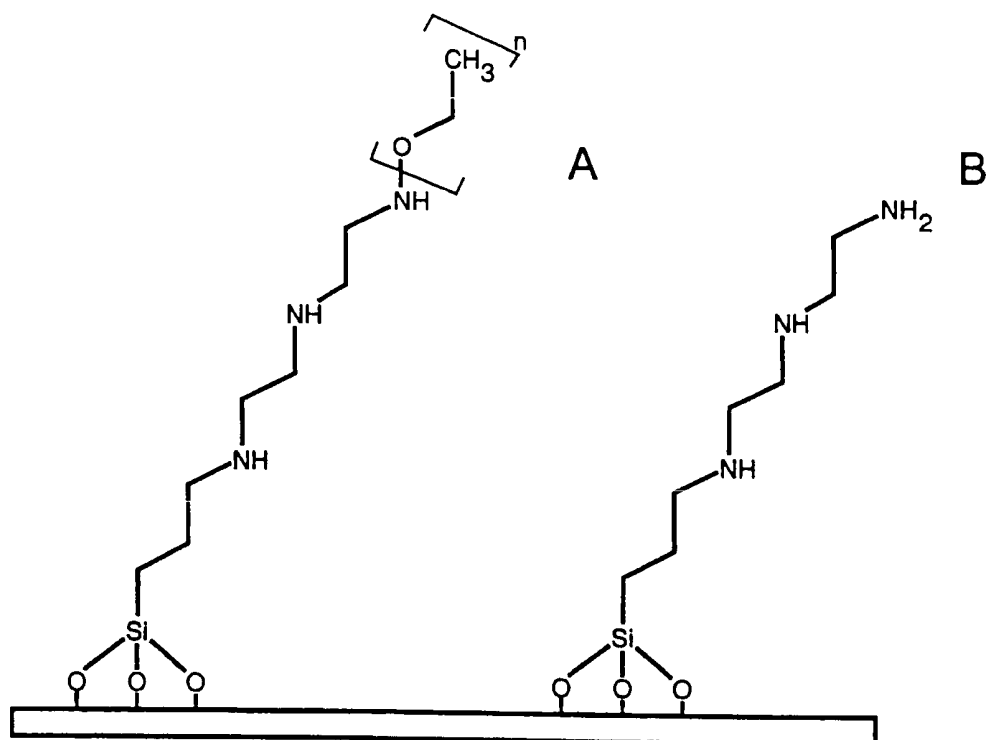
Figure 43

Figure 44
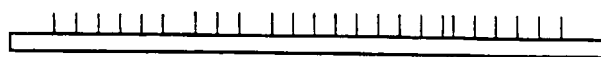
Oxidized plastic or clean glass has hydroxyl groups on the surface
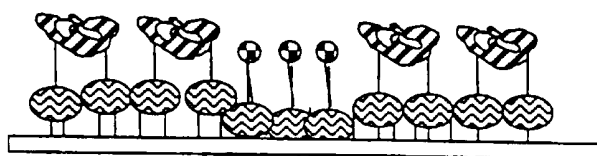
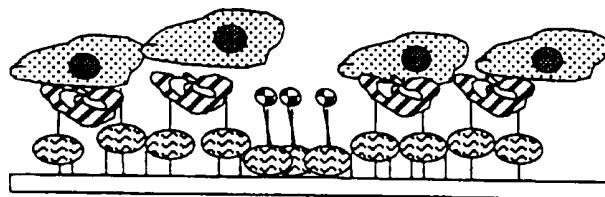React substrate with an aminosilane

Apply stencil

Vapor or solution deposit tresyl-PEG

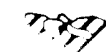Remove mechanical mask and apply cell adhesive molecules

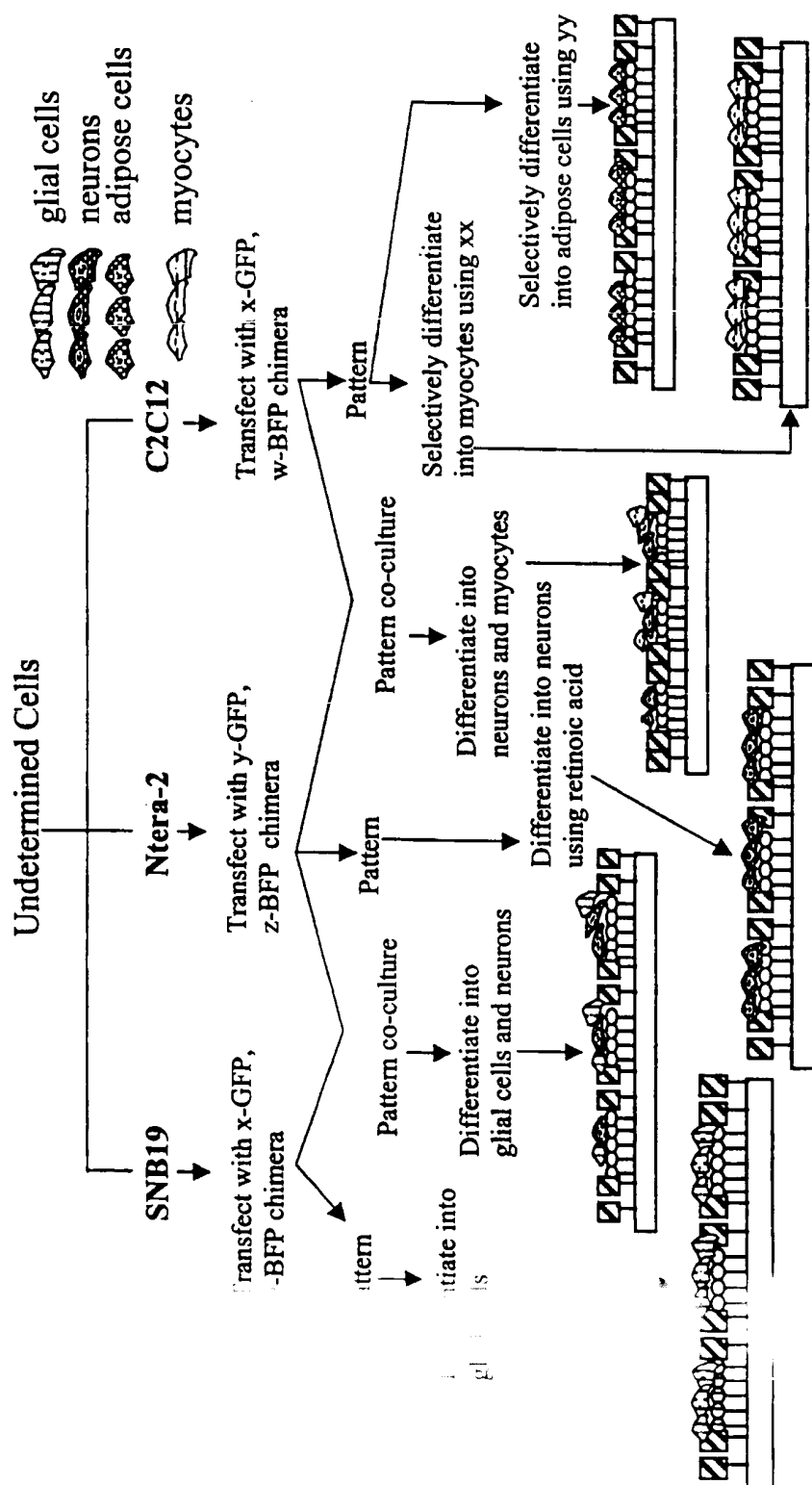Apply cells, they will adhere to previously masked areas

Aminosilane

Tresyl-PEG

SUBSTITUTE SHEET (RULE 26)

Figure 45

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 776 748 A (WHITESIDES GEORGE M ET AL) 7 July 1998 (1998-07-07) column 2, line 43 -column 9, line 52 ---	15
A	DEWEZ J -L ET AL: "Adhesion of mammalian cells to polymer surfaces: from physical chemistry of surfaces to selective adhesion on defined patterns" BIOMATERIALS,GB,ELSEVIER SCIENCE PUBLISHERS BV., BARKING, vol. 19, no. 16, August 1998 (1998-08), pages 1441-1445, XP004161407 ISSN: 0142-9612 the whole document ---	1-25

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2000	18/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Muller, T

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 15223 A (UNIV LOUVAIN ;DEWEZ JEAN LUC (BE); LHOEST JEAN BENOIT (BE); DETRAI) 23 May 1996 (1996-05-23) page 3, line 20-29; figure 1 ---	1-25
A	RAHUL SINGHVI,AMUT KUMAR,GABRIEL P. LOPEZ, GREGORY N. STEPHANOPOULOS, DANIEL I.C.WANG, GEORGE M. WHITESIDES, DONALD E. INGBER: "Enineering Cell Shape and Function" SCIENCE, vol. 264, 29 April 1994 (1994-04-29), pages 696-698, XP000919113 cited in the application the whole document ---	1-25
A	GEORGE B. SIGAL, CYNTHIA BAMDAD,ALCIDE BARBERIS,JACK STROMINGER,GEORGE M. WHITESIDES: "A self-assembled monolayer for the binding and study of Histidine-Tagged proteins by Surface Plasmon Resonance" ANALYTICAL CHEMISTRY, vol. 68, no. 3, 1 February 1996 (1996-02-01), pages 490-497, XP000916112 cited in the application the whole document -----	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5776748	A	07-07-1998	US	5976826 A	02-11-1999
WO 9615223	A	23-05-1996	BE	1008955 A	01-10-1996
			EP	0800574 A	15-10-1997
			US	5962136 A	05-10-1999